Identifier et traiter la tendinopathie modérée des tendons de queue de rat *ex vivo*

Identifying and treating moderate tendinopathy in rat tail tendons *ex vivo*

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PhD thesis

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Abstract

Background: Tendinopathy, a general condition of tendon disorder, is common among people specially athletes and workers. Tendinosis, an asymptomatic tendon degeneration without inflammation, is a frequent form of tendinopathy which can be chronic and difficult to successfully treat in long term. The focus of this research study is on this type of tendinopathy. The proper strategy to prevent or treat chronic tendinopathy (CT) is still unclear. Understanding the pathology behind CT may help finding proper treatments. Although significant advances have been made to understand the tendinopathy mechanisms, this condition is still poorly understood.

Studying CT not only is necessary in order to provide further information about etiology and pathogenesis, but also to improve healing strategies. Although many treatment strategies have been suggested for CT disorders, none of them has satisfying results.

Subject: Therefore, the main subject of this study was to characterize a CT model *ex vivo*, which represents the level of degeneration which is not too early to be diagnosed and not too late to be treated. This is a transient stage between early and advanced tendinopathy. We refer to this stage as moderate tendinopathy.

After creating moderate tendinopathy, the efficacy of two existing treatments, i.e. matrix metalloproteinase inhibitor (MMPI) and platelet-rich-plasma (PRP) injections, in combination and alone, on healing moderate tendinopathy model was investigated.

Methods: The *ex vivo* model was characterized by using stress deprivation. Rat tail tendons (RTTs) were cultured without loading for 0 (control), 2, 4, 6, or 10 days. RTTs were subjected to traction testing as well as to histopathological, biochemical, and viability assays at the end of their culture time. Then, the *ex vivo* moderate tendinopathy model was further cultured for 6 days with or without receiving treatment(s). The treatments included low static loading, alone or in combination with: narrow spectrum MMPI (NI), broad spectrum MMPI (BI), PRP, PRP + NI, PRP + BI. Again, RTTs were subjected to traction

testing as well as histopathological, biochemical, and viability assays at the end of their culture time.

Results: There were moderate degradative changes in the properties of RTTs at day 4 of SD. They included: increases in the space between fibers, cell density, and collagen tortuosity as well as a decrease in collagen density and elongation of cell nuclei. No changes in the stress at failure of RTTs were observed at this time point. Advance degradative changes occurred at later time points, i.e. at days 6 and 10 of SD. They included: more increases in the space between fibers, and collagen tortuosity as well as a decrease in collagen density and elongation of cell nuclei. There was also a significant decrease in stress at failure of RTTs at days 6 and 10 of SD compared to fresh tendons.

Moreover, our results showed that PRP + NI added to low static loading, improved mechanical and histological properties of moderately damaged tendons comparing to other treatments, or untreated tendons. Stress at failure of RTTs treated with PRP + NI was improved comparing to 10-day SD tendons ($p=0.01$). PRP + NI also improved fiber density, nucleus shape, and space between fibers of RTTs when compared to 10-day SD RTTs ($p<0.05$).

Conclusions: The simple *ex vivo* model characterized in this study is useful to study the progression of CT, and to investigate the efficacy of potential treatments to stop or reverse the progression of the pathology. Moreover, using this model, we have introduced PRP and NI combination therapy as a potentially efficient treatment for CT.

Key words: tendinopathy, stress deprivation, matrix metalloproteinase inhibitor, platelet-rich-plasma.

Résumé

Contexte: La tendinopathie, une condition générale qui affecte le tendon, est fréquente, spécialement chez les athlètes et les travailleurs. La tendinose, une dégénérescence du tendon sans inflammation, est une forme fréquente de tendinopathie qui peut être chronique et difficile à traiter à long terme. Cette étude est axée sur ce type de tendinopathie. La stratégie appropriée pour prévenir ou traiter la tendinopathie chronique (TC) n'est toujours pas claire. Mieux comprendre la pathologie de la TC contribuera à améliorer les traitements appropriés. Bien que des progrès significatifs aient été réalisés pour comprendre les mécanismes de tendinopathie, cette condition est encore mal comprise.

L'étude de la TC n'est pas seulement nécessaire pour fournir de plus amples informations sur l'étiologie et la pathogenèse, mais aussi pour améliorer les stratégies de guérison. Bien que de nombreuses stratégies de traitement aient été suggérées pour la TC, aucune n'a donné de résultats satisfaisants.

Objet: L'objet principal de cette étude était donc de caractériser un modèle *ex vivo* de TC, qui représente le niveau de dégénérescence qui n'est pas trop précoce pour être diagnostiqué et pas trop avancé pour être traité. C'est un stade transitoire entre les tendinopathies précoces et avancées. Nous appelons ce stade *tendinopathie modérée*.

Après avoir créé une tendinopathie modérée, l'efficacité de deux traitements existants, à savoir l'ajout d'inhibiteurs de métalloprotéinases de la matrice (IMPMs) et de plasma riche en plaquettes (PRP), en combinaison et individuellement, sur le modèle de tendinopathie modérée a été étudiée.

Méthodes: Le modèle *ex vivo* créé par la privation de charge mécanique a été caractérisé. Les tendons de queue de rat (TQRs) ont été cultivés en absence de charge mécanique pendant 0 (témoin), 2, 4, 6 ou 10 jours. Les TQRs ont été soumis à des tests de traction ainsi qu'à des analyses histogiques, biochimiques et de viabilité à la fin de leur temps de culture. Ensuite, le modèle de tendinopathie modérée *ex vivo* a été cultivé pendant 6 jours

avec ou sans traitement. Les traitements comprenaient une faible charge statique, seule ou en combinaison avec: IMPM à spectre étroit (IÉ), IMPM à large spectre (IL), PRP, PRP + IÉ, PRP + IL. Encore une fois, les TQRs ont été soumis à des tests de traction ainsi qu'à des analyses histologiques, biochimiques et de viabilité à la fin de leur temps de culture.

Résultats: Il y avait des changements modérés dans les propriétés des TQRs au jour 4 de la privation de chargement mécanique. Ils comprenaient : l'augmentation de l'espace entre les fibres, de la densité cellulaire et de la tortuosité du collagène, ainsi qu'une diminution de la densité du collagène et de l'élongation des noyaux cellulaires. Aucun changement dans la contrainte à la rupture des TQRs n'a été observé à ce moment. Les changements de dégradation avancés se sont produits à des moments ultérieurs, à savoir aux jours 6 et 10 de privation de charge mécanique. Ils comprenaient : une augmentation de l'espace entre les fibres et de la tortuosité du collagène, ainsi qu'une diminution de la densité du collagène et de l'élongation des noyaux cellulaires. Il y avait également une diminution significative de la contrainte à la rupture des TQRs aux jours 6 et 10 de privation de chargement par rapport aux tendons frais.

De plus, nos résultats ont montré que le traitement PRP + IÉ, ajoutés à la faible charge statique, améliorait les propriétés mécaniques et histologiques des tendons modérément endommagés par rapport à d'autres traitements ou aux tendons non traités. La contrainte à la rupture des TQRs traités avec PRP + IÉ a été améliorée par rapport aux tendons privés de chargement au jour 10 ($p = 0,01$). Le traitement PRP + IÉ a également amélioré la densité des fibres, la forme du noyau et l'espace entre les fibres des TQRs par rapport aux TQRs privés de chargement au jour 10 ($p < 0,05$).

Conclusions: Le modèle simple *ex vivo* caractérisé dans cette étude est utile pour investiguer la progression de la TC et pour étudier l'efficacité des traitements potentiels pour arrêter ou inverser la progression de la pathologie. De plus, en utilisant ce modèle, nous avons introduit la thérapie combinée PRP et IÉ comme traitement potentiellement efficace pour la TC.

Mots clés: tendinopathie, privation de charge mécanique, inhibiteur de métalloprotéinases de la matrice, plasma riche en plaquettes.

Chapter 1. Introduction

Tendons are those connective tissues which connect muscle to bone. They consist of two main parts: the extracellular matrix (ECM) and cells (tenocytes) which are, respectively, the inert and active components of tendons. These two components are in a close and bidirectional interaction. However, the mechanical behavior of the tendon is mostly devoted to the ECM, and cells are considered as responsible for tissue remodeling [42].

ECM is mainly made up of water ($\sim 70\%$). The dry mass of ECM consists mostly of collagen, elastin, and proteoglycans. Collagen is the most abundant protein in ECM. It provides tendon strength against applied tensile load. Collagen parallel alignment along the tendon enables it to resist tensile load in this direction.

Tenocytes: tenocytes, which are responsible for production of the ECM, have an elongated shape when observed in the tendon's longitudinal orientation[71]. Whereas in cross-sectional view, they appear as star-shaped[64]. Tenocytes are sparsely distributed between collagen fibers with their processes making an extensive network inside ECM[114]. They are highly responsive to their micro-environmental changes, such as mechanical loads or local stimuli[28]. For further details regarding tenocytes mechano-responsiveness see section 2.1.2.

Collagen in tendons adopts a hierarchical structure such as displayed in Figure 1.1. Collagen molecules unite into collagen fibrils, collagen fibers*, subfascicles (primary bundles), fascicles (secondary bundles) and tertiary bundles[53]. Primary, secondary and tertiary fiber bundles are covered by a thin layer called endotenon and the whole tendon is surrounded by another thin layer called epitenon[91].

* There have been some misunderstandings in literature regarding using "fiber" and "fibril". In some texts, these two terms have been used interchangeably.

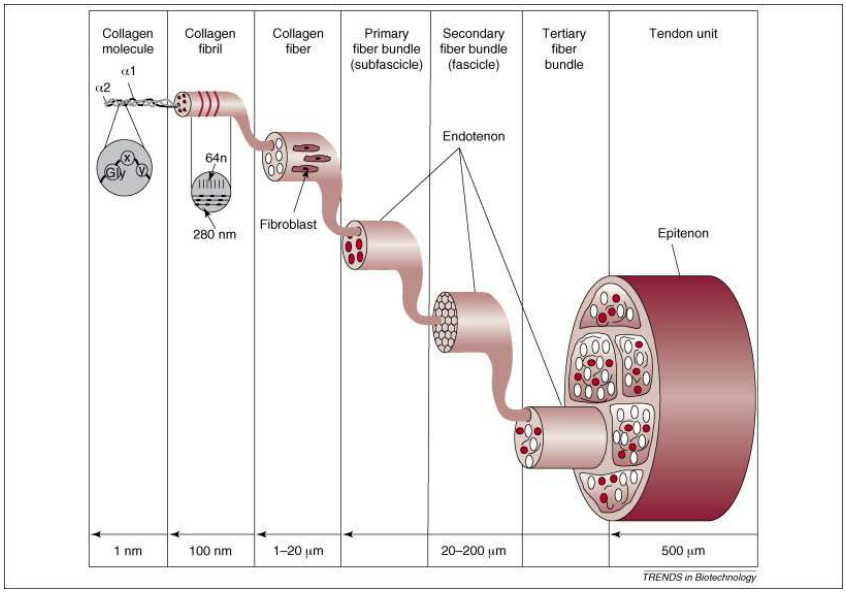

Figure 1.1 Schematic structure of a normal tendon[49]

Tendon disorders are common health problems. For example, 1 of every 10 people and 1 of every 2 runners experience Achilles tendon disorder before the age of 45[98]. The consequences of this medical problem include pain, disability, early retirement from sport and work, mental distress, health-care costs, etc.

It should be noted that there is confusion in literature about terminology of tendon disorders. One term which has been used to address tendon problems is "tendinopathy". Tendinopathy is a general term for tendon disorders with no assumption of underlying pathology. Traditionally, it was thought that underlying pathology of tendon disorders was due to inflammation[2,27,83]. This condition, i.e. tendon disorder accompanied by inflammation, is referred to as "tendinitis". However, later, "non-inflammatory" or "degenerative" theory was considered to be the reason behind CT, or overuse injuries[83]. The term used to describe this condition i.e. degenerative condition of tendon without accompanying inflammation is "tendinosis"[83,84]. These three terms have often been used interchangeably which is inappropriate. We notify that in this research, the focus is on a **tendinopathy model of chronic injury without inflammation**.

Some important features of CT *in vivo* based on literature include:

Clinical features: activity-related pain, tenderness, and impaired performance[43].

Histological features: hyper-cellularity, loss of cytoplasm elongated shape, rounder nuclei, neovascularization, disruption in collagen organization and orientation, decreasing collagen fibers size and increasing cross sectional area (CSA)[43,55].

Biochemical features: changes in gene expression and activity of biomolecules and proteins such as collagens and matrix metalloproteinases (MMPs), enzymes which degrade ECM (for further details, see Appendix). For example, it has been suggested that there is an increase in MMPs level in tendinopathic tendon[5]. However, these changes are often model-dependent. In other words, they depend on various factors such as species (human, rat, rabbit, etc), body parts (Achilles tendon, hamstring tendon, tail tendon, etc), and type of pathology (acute, overuse, stress deprivation, etc).

Despite the prevalence of tendon problems, they are difficult to treat successfully[84,86]. Historically, treatment strategies were largely focusing on targeting inflammation such as rest, corticosteroid injection, or nonsteroidal anti-inflammatory drugs (NSIADs). However, since there is little or no inflammation in CT[2,83], the results of these treatments on CT are usually unsatisfactory, and there is possibility of recurrence of symptoms[43]. Currently new promising treatments (see section 2.2) are being developed, but there is still little evidence to support their use.

To find an optimal healing strategy, understanding the pathology behind tendinopathy is crucial. Using animal models is essential for in-depth studies on this subject. Therefore, in the first part of our literature review (Chapter 2), available models relating to CT are described.

Models are not only beneficial for studying features and causes of CT, but they also enable studying potential treatments. Hence, available treatments for tendinopathy will be reviewed in the second part of our literature review. Thereafter, we will introduce the selected treatments to be studied in this research.

Two articles based on the findings of this research are presented in Chapters 3 and 4. In the first article (Chapter 3), a simple *ex vivo* model to create moderate CT has been characterized. The second article (Chapter 4) presents the results of applying two recent treatments (PRP, and MMPI) in combination and alone on this *ex vivo* tendinopathic model. Lastly, in Chapter 5, concluding remarks from the results of this project and suggestions for further work are outlined.

Chapter 2. Literature review

2.1. Currently available *ex vivo* models for tendinopathy.

There are numerous pathogenetic mechanisms behind tendinopathy. Moreover, tendons from different body parts, thus subjected to different mechanical and biological conditions, are affected by tendinopathy. Therefore, it is unlikely that only one suitable model exists to represent all tendinopathy conditions.

Tendinopathy models for the study of pathogenesis and the evaluation of potential treatments are developed either *in vivo* or *ex vivo*. *In vivo* models are those inducing tendinopathy in a live animal. *Ex vivo* models include those using a living tendon kept outside of the body under culture conditions.

In vivo models are essential, because they enable us to have a more complete understanding of tendinopathy[27]. *Ex vivo* models are also critical, because they allow under specific, controlled conditions (e.g. defined media, defined loading protocol, …), the study of the pathological basis as well as of drug efficacy. Since *in vivo* models are costly and time-consuming, we decided to initially characterize an *ex vivo* tendinopathy model to verify our hypotheses. In the following sections, the current *ex vivo* chronic tendinopathy (CT) models will be explained. Finally, we conclude with the method we selected to create CT in this research.

2.1.1. *Ex vivo* models of tendinopathy

Ex vivo models of tendinopathy allow for great control over experimental conditions[27], for example loading conditions or composition of culture media. *Ex vivo* models mainly use cyclic loading, and stress deprivation (SD) to induce tendinopathy in harvested tissues. In these models, there is no need to interact recurrently with animals, to make them exercise for example. Therefore, these models may be cheaper and less time consuming.

Cyclic loading

This model has been developed to simulate the daily repetitive loading on live tendons. For example, Cousineau and Langelier[22] used two loading conditions: i.e. 1.2% strain for underuse, and 1.8% strain for overuse. For both loading conditions, they have reported a decrease in the peak stress of the cyclically loaded rat tail tendons (RTTs) after 24 hours as well as changes in the ECM after 10 days, mainly for the underuse condition.

In another study[26], live avian flexor digitorum tendons were cyclically loaded to either 3 (low), or 12 MPa (high), for either continuously 24 hours (short), or 2h/day for 12 days (long) at 1 Hz. The results revealed a decrease in maximum load at failure (N) of tendons loaded with the following regimens: low/long, high/short and high/long. Maximum stress at failure (MPa) of tendons which were loaded high/short or high/long also decreased significantly.

Cellular deformation as a result of cyclic loading has been reported in a study on rat tail tendons[7]. The results of this study demonstrated that exposing tendons to 2%, 4% and 6% strain results in a deformation in cell nuclei.

Cellular response to cyclic loading has also been the subject of some studies. For example, an increase in the level of PGE_2 as a result of increasing loading duration of avian flexor digitorum tendons has been reported[26]. Moreover, a decrease in MMP-1 levels of cyclically loaded rat tail tendons has been demonstrated[50].

Stress deprivation

Ex vivo SD also could cause tendon degeneration. This model has frequently been used to study tendinopathy. In the following paragraphs, two studies have been explained to introduce the general results. For further studies and results, please see Table 3.1 (chapter 3).

Arnockzky *et al* [5] developed a stress-deprived model by incubating live tendons without mechanical load for 7 days. This model produced a reduction in the intimate contact of cell

and its pericellular matrix; a reduction in collagen fibril packing; a reduction in ultimate stress, strain at ultimate stress, tensile modulus; and finally, an increased MMPs levels.

In a study by Lavagnino *et al* [49], *ex vivo* stress deprivation resulted in a reduction in tensile properties without changes in collagens.

For further details about the effect of stress deprivation on tendon properties *ex vivo*, please refer to Table 3.1 (chapter 3).

2.1.2. Pathogenesis behind tendinopathy induced by stress deprivation and cyclic loading

To address the reason of degradative effects of stress deprivation and cyclic loading on tissues, we should first consider that tendon is a mechanosensitive tissue which responds to loading in an adaptive manner[45]. Mechanical loading on tendons can be sensed by tendon cells via mechanotransduction mechanisms. Mechanotransduction mechanisms, by which mechanical stimulations are converted into biochemical signals, are mediated by cell deformation, nucleus deformation, cytoskeleton, stretch activated channels, and primary cilium[100] (Figure 2.1). According to Arnoczky *et al* [6], the character of cell response (catabolic vs. anabolic) to the perceived load depends on its threshold of sensitivity to external load. It has been demonstrated that the preset level of cell sensitivity to the applied load, which is called *mechanostat set point of the cell*, is regulated by cells through their ability to produce internal tension. This tension may help cells to maintain their *homeostatic state of stress*[6]. If the applied load is less than this set point, the response of cells will be catabolic, while if the load is higher than *cell mechanostat set point*, cell response will be anabolic.

The degradative effects of stress deprivation, as a result of catabolic response of the cells, could be explained by this theory. Uchida *et al* [96] reported a simultaneous over-expression of cytokines such as IL-1b and TNF-a following stress deprivation. These signaling molecules are well-known to stimulate MMPs-1 and -3 in fibroblasts. They addressed these ECM-degradative enzymes as the reason of destructive effects of stress deprivation.

However, they did not explain the reason of the catabolic cell response, which seems to be related to *mechanostat set point of the cells*.

In cyclic loading, beside cell response, mechanical degradation of ECM should also be taken into account. Since tendons have a viscoelastic behavior, the factors which mediate mechanical degradation include: the amount, the frequency and the rate of applied load, and rest periods between loading episodes. Mechanical degradation would lead to tendinopathy if the anabolic response is insufficient to repair tissue damage. This would lead to the accumulation of micro tears in collagen fibers. The partial damages of collagens unable them to properly transfer the external load to cells; as a result, tendon cells undergo understimulation. It means that their perceived load is less than their *mechanostat set point*. Therefore, their response would be catabolic, and they produce degeneration which may eventually lead to tendinopathy[4]. Although the etiology is likely more complicated than this, accumulation of microdamages as a result of fatigue loading has been considered a significant reason for overuse injuries[26].

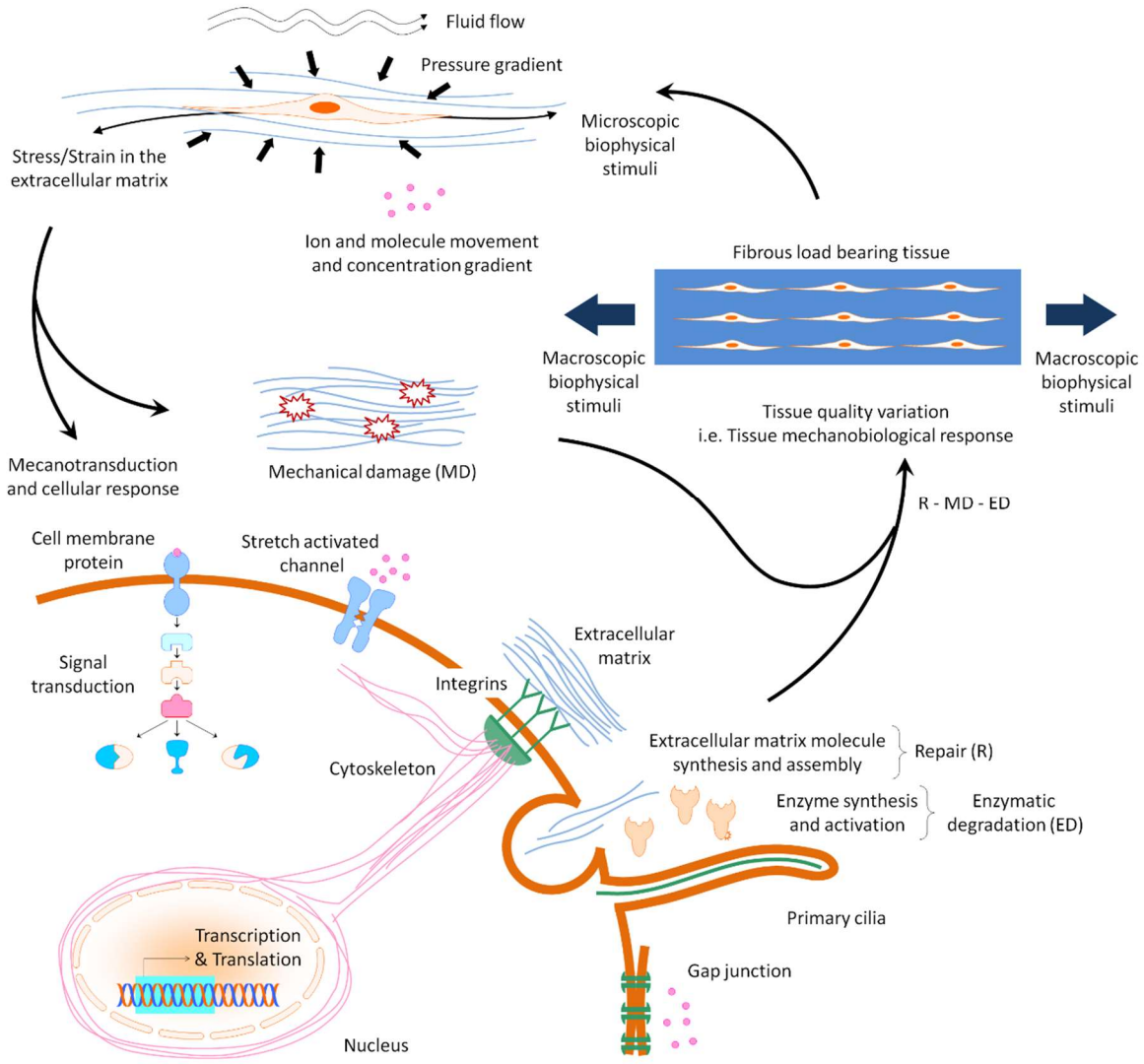

Figure 2.1 Tissue mechanobiological response including cellular response and extracellular matrix mechanical damage. Note the mechanotransduction mechanisms by which cell senses the mechanical loading.

2.1.3. The preferred CT model

Among all *ex vivo* tendinopathy models, we chose SD to characterize CT in our laboratory. We use SD because it could be achieved by using a simple experimental setup. Therefore, there is no need to use the bioreactor which is not very stable and also has the limitation of number of samples per experiment (4 tendons). Moreover, preparing the samples in this model is less expensive in terms of cost and length of experiment. This is in concordance to the objectives of this research study: because we need to control multiple factors in each experiment, this model allows for having enough samples simultaneously. Moreover, since these samples could all be extracted from the same animal, the risk of inter-animal variability is also reduced, as well as the number of sacrificed animals.

2.2. Current healing strategies available to treat tendinopathy

Despite the prevalence of tendinopathy, the available healing strategies are not completely satisfactory[43]. Traditional treatments focused on anti-inflammatory drugs such as corticosteroid injections and non-steroidal anti-inflammatory drugs (NSAID) drugs. However, it may not be the most appropriate target[2], because it has been suggested that there is little or no inflammation in CT[2]. Even in tendinitis, there is debate on whether blocking the inflammatory response will be helpful or not[84]. Currently, several treatments, (including eccentric exercises, pharmacological treatments, etc) have been introduced to heal tendinopathy. However, there is little high-quality studies to confirm their clinical use[21,72,90]. A summary of current non-surgical tendinopathy treatments is demonstrated in Table 2.1.

Table 2.1 The rational and efficacy of available tendinopathy treatments[2,43,84,86]

Treatments	**Rationale or Function**	**Efficacy and State-Of-The-Art**
Non-pharmacological treatments		
Ice[43,86]	Reduction of acute inflammation, because of the reduction in blood flow and metabolism rate	Good efficacy on short-term pain relief No efficacy on long-term pain relief Little research has been done on it
Therapeutic ultrasound[2,43,84,86]	Thermal effects on tissue, cell activity stimulation and blood flow increase	Little or good efficacy on short-term pain reduction No efficacy on long-term pain reduction Controversy in the literature and little evidence to support its use
Laser therapy[2,84,86]	Possible analgesic effects and some unknown effects on cell activity	Little or good efficacy on short-term pain reduction No efficacy on long-term pain reduction Contradictory results

Friction massage[2,43,84]	Cell activity stimulation and blood flow increase	Little or good efficacy on short-term pain reduction No efficacy on long-term pain reduction Controversy in the literature and little evidence to support its use
Soft tissue mobilization[2,84]	Cell activity stimulation and blood flow increase	Little or good efficacy on short-term pain reduction No efficacy on long-term pain reduction Controversy in the literature and little evidence to support its use
Orthotic devices[43,84,86]	Correcting a static disorder, removal of precipitating factors and prevention of reinjury	Good efficacy on short-term pain relief No efficacy on long-term pain relief Little evidence to support their effectiveness

Eccentric exercise[2,43,84,86]	Promote regeneration of normal tissue structure, probably through an effect on cell activity and matrix remodeling	Good efficacy on short-term pain relief and long-term pain relief It is currently considered to be the most efficient treatment for tendinopathy, although some studies are contradictory
Extracorporeal Shock Wave Therapy (ESWT)[2,43,84,86]	Promote tissue healing by stimulation of cell activity, and neovascularization, induction of growth factors (TGF-β1 and IGF-1), increased tenocyte proliferation and collagen synthesis	Very good efficacy on short-term pain relief and long-term pain relief Some evidence to support its use in calcific tendinopathies of the rotator cuff No clear evidence, or little evidence (controversial information) supporting its use in the treatment of chronic tennis elbow, medial epicondylitis, Achilles and patellar tendinopathies
Pharmacological treatments		
Nonsteroidal Anti-inflammatory Drugs (NSAIDs)[2,43,84,86]	Reduction of inflammation through inhibition of inflammatory factors synthesis	Good efficacy on acute pain No efficacy on long-term pain relief Researches in acute tendon injury model showed NSAID administration did not

		prevent collagen degradation or loss of tensile force in tendons Its use is still controversy regarding the lack of existence of inflammation in degeneration
Corticosteroid injections[2,43,84,86]	Reduction of inflammation by inhibition of the synthesis of cytokine genes and proinflammatory factors	Good efficacy on short-term pain relief Little or no efficacy on long-term pain relief Mixed results Corticosteroids can cause spontaneous tendon rupture No evidence to prove its efficiency for chronic tendinopathy
Heparin[84,86]	Effect on tendon blood flow; possibly results in improved healing	In a review in 2006, it was mentioned that it has some constructive effects, but more recent animal study found heparin had a degenerative effect

		It has not been mentioned in the more recent reviews (2008, and 2011), therefore may be it is no more an option
Dextrose[84]	Possibility of releasing growth factors (GFs) and therefore local tissue proliferation	Since there is no control data nothing can be said It has not been mentioned in the reviewed literature as a potential treatment since the 2006 review article
MMP inhibitors[43,84]	Promoting healing by inhibiting the enzymes which break down tendons, i.e. MMPs. Because it has been established that using conventional treatments with the effect of inflammatory suppression May not fully inhibit MMP-based tendon degradation	Very good efficacy on short-term pain relief and long-term pain relief In clinical trials, aprotinin was used as a broad-spectrum MMP inhibitor Good clinical improvement mild-Achilles tendinopathy patients were treated more successfully than patellar Aprotinin may cause anaphylaxis, more studies are needed

Injections of blood or PRP[2,43,84,86]	Contains growth factors (e.g. transforming growth factor β and platelet-derived growth factor) that promote matrix synthesis and tissue repair	Little or no efficacy on short-term pain relief Very good efficacy on long-term pain relief There have been only a few clinical studies in recent years with good results, but *in vitro* studies have promising results. Other controlled studies are needed
Sclerosant injections[2,43,84,86]	Blocks tendon blood flow (targeting neovascularization and associated pain generating nerve in-growth)	Very good efficacy on short-term pain relief and long-term pain relief In clinical trials polidocanol was used as sclerosant agent Some clinical studies report goodshort- and/or long-term result with an increase in strength and a decrease in pain Other studies needed to evaluate its safety, efficacy, and combination with other drugs

Topical glyceryl trinitrate[2,43,84,86]	Enhances collagen synthesis	Very good efficacy on short-term pain relief Excellent efficacy on long-term pain relief Few clinical trials, reported benefit on patient-determined pain, function, and loss of symptoms on Achilles tendinopathy, chronic supraspinatus tendinopathy and lateral elbow tendinopathy
Polysulphated glycosaminoglycan[84]	Inhibition of inflammation, possibly also acting to inhibit metalloproteinase enzyme activity	A number of studies suggesting that injection of glycosaminoglycan polysulphate may lead to an improvement in disease of the human Achilles and equine superficial digital flexor tendon. It has not been mentioned in the recent reviews (2008, and 2011), therefore may be it is no more an option
Botulinum toxin A (BTA) injections[43]	Paralysis caused by BTA involves a reduction in tensile stress on the enthesis	Very good efficacy on short-term pain relief

		Good efficacy on long-term pain relief Few articles from last years have considered the possibility of making botulinum toxin injections (BTA) to treat epicondylitis
Stem-cell or gene therapy[2,43,84,86]	Stem cells will be isolated and located in the area of injury or degeneration. Then with local signaling or some exogenous factors, they will be stimulated to differentiate to particular cells	Unknown efficacy on pain relief There are promising *in vitro* results for using stem-cell or gene in treatment of tendinopathy. Animal studies suggest that gene therapy may also improve the capacity of the injured tendon to heal

Among all potential treatments, we selected platelet-rich plasma (PRP) and matrix metalloproteinase inhibitors (MMPIs) to apply in our laboratory. To explain this choice, we refer to the following model for tendon mechanobiological response (TMR) (Figure 2.1) as: TMR = Repair – (Mechanical degradation + Enzymatic degradation)[22].

This model highlights that the combination of these treatments might be beneficial to simultaneously **promote regeneration** and **inhibit enzymatic degeneration**. First, PRP would enhance tendon repair through the release of a variety of autologous growth factors (GFs) playing a key role in tendon repair mechanisms[68]. Second, MMPIs would inhibit proteolytic degradation of connective tissues mediated by MMPs. However, in our knowledge the combination of these two treatments has not been studied so far.

In the following section these two healing strategies are explained with more details.

2.3. MMPIs and PRPs: The selected healing strategies to apply in *ex vivo* created tendinopathy models

2.3.1. MMPIs: general information

Natural and synthetic MMPIs

MMPIs are enzymes inhibiting the activity and/or expression of MMPs. MMPs are ECM proteolytic enzymes mediating ECM turnover. ECM equilibrium is mediated by, *inter alia*, a balance between the levels of MMPs and their natural inhibitors, called tissue inhibitors of matrix metalloproteinases (TIMPs).

Dysregulation of the balance between the levels of MMPs and TIMPs interrupts ECM equilibrium. High levels of MMPs could cause tendon degeneration and rupture. Therefore, inhibiting MMPs has been considered as a therapy option for tendinopathy[5,73].

MMPIs could be endogenous such as TIMPs, or exogenous such as synthetic MMPIs. TIMPs are divided into four categories: TIMP1, TIMP2, TIMP3, TIMP4. They inhibit all MMPs, however, the ability of some of TIMPs to inhibit some specific MMPs needs to be elucidated

in future studies. TIMPs function is not limited to inhibition of MMPs. They also play roles in regulation of angiogenesis and cellular proliferation[78].

Although TIMPs have stronger inhibitory effect than synthetic MMPIs, to our knowledge, they have never been used as therapeutic agents to promote tendon healing *ex vivo* or *in vivo*. Before further using them as treatment in diverse pathologies, it has been suggested to deepen our understanding of TIMPs' activities (other than inhibitory ones), since they may cause some unwanted side effects[10]. Moreover, their broad-spectrum inhibitory effect makes them undesirable in studies with specific MMPs in target. However, TIMPs could be modified in order to retain their inhibitory effect without their other unwanted biological activities. In this way, they may be applied for therapeutic benefits in future[10].

It should be noted that TIMPs have been used as therapeutic agent in some studies of other disease. For instance, Li *et al* have shown promising results of using recombinant TIMP-2 in cancer treatment[52].

Synthetic MMPIs have been used as therapeutic agents in MMP-mediated disease. There are several studies with using synthetic MMPIs to promote tendon healing *ex vivo* and *in vivo*. Some of them will be presented in the following sections.

The effect of MMPIs on tendons in *ex vivo* and *in vivo* animal studies

Excessive action of MMPs may lead to degeneration and weakening of ECM. Therefore, in this condition, drugs that can decrease the MMPs activity, and therefore ECM degeneration, may play a significant role in promoting tendon healing[5]. In some *ex vivo* and *in vivo* studies, MMPIs were used to affect MMP activity and prevent or reverse the process of MMP-mediated ECM degeneration in tendons. Table 2.2 summarizes the result of MMPI application on tendons in some studies *ex vivo*, and *in vivo*. It should be noted that most of the studies were conducted on surgically-induced tendinopathy, i.e. partially or completely sectioning the tendon using a scalpel and then stitching damaged tendons or leaving them unsutured, models which do not reflect CT.

Table 2.2 Data extracted from MMPI studies. N/A stands for data not available.

Reference	**Model**	**Experiment**	**Results**
Bedi et al, [13] 2002	• *In vivo* • Rat, male, 250-300g • Rotator cuff • Surgically induced damage	***Treatment*** • Doxycycline • @ preoperative day (D-1), or @ 5 and 14 days postoperative (D+5 and D+14) until the time of sacrifice ***Analysis***: • Postoperative day (POD) #5, #8, #14, #28	***Biomechanical*** • D-1: Increased load to failure at 2 wks compared to control, (and no difference at day 8) • D+5: Increased load to failure at 2 wks compared to control, (and no difference at day 8) • D+14: No difference ***Histological*** • D-1: Improved collagen organization at POD#5, #8, #14; • D+5: Improved collagen organization at POD#14; • D+14:No difference ***Biochemical*** • D-1: Reduced MMP13 activity at POD#8 compared to control,

Arnoczky et al, [5] 2007	• *Ex vivo* • Rat, adult • Tail tendon • Healthy	***Treatment*** • Doxycycline or Ilomastat • @ day 0 ***Analysis***: • 7 days after first MMPIs application	***Biomechanical*** • Improvement in ultimate stress, tensile modulus, and strain at ultimate stress with either of MMPIs compared to 7-day SD ***Histological*** • Limited the alteration in the pericellular matrix shape, and loss of cell-matrix contact with either of MMPIs; ***Biochemical*** • Reduction in MMP13 activity with either of MMPIs, • No effect on MMP13 protein synthesis with either of MMPIs
Demirag et al, [25] 2005	• *In vivo* • Rabbit, 3.5-4 kg • Anterior cruciate ligament • Surgically induced damage	***Treatment*** • A-2-macroglobulin • @ day 0 and 1 after surgery ***Analysis***: • 2 and 5 weeks post-surgery	***Biomechanical*** • Greater load to failure at both 2 and 5 weeks ***Histological*** • Dense, more mature, perpendicular collagen fibers ***Biochemical*** • Decreased MMP-8 activity

Kessler et al, [44] 2014	• *In vivo* • Rat, male, 300-400 g • Achilles • Surgically induced damage	***Treatment*** • Doxycycline • Starting @ preoperative day, continued up to 4 weeks ***Analysis:*** • 2 or 4 weeks for histology; 3 weeks for biomechanical properties	***Biomechanical*** • Enhanced biomechanical properties ***Histological*** • Improved collagen fiber organization ***Biochemical*** • Reduced MMP activity
Nquyen et al, [70] 2017	• *In vivo* • Rat, male, 2-3 months old • Achilles • Surgically induced damage	***Treatment*** • Doxycycline • Daily administration of MMPI one day after injury ***Analysis***: • 1.5, 3, 6, 9 weeks after injury	***Biomechanical*** • Accelerated recovery in biomechanical properties (i.e., greater equilibrium modulus, higher dynamic modulus and lower creep strain) ***Histological*** • Improved tissue organization, fiber alignment, and decreased fiber dispersion ***Biochemical*** • Decreased MMP3 expression at 9 wks
Bedi et al, [14] 2010	• *In vivo* • Rat, male, 250-300 g	***Treatment*** • A-2-macroglobulin • @ day 0	***Biomechanical*** • No change in load to failure, or stiffness ***Histological***

	• Rotator cuff • Surgically induced damage	***Analysis***: • 2 or 4 weeks after surgery	• Greater collagen organization at 4 wks; but not at 2 wks • Reduction in collagen degradation at both 2, and 4 wks ***Biochemical*** • None
Orner et al, [74] 2016	• *In vivo* • Mouse, female, 8-10 weeks • Flexor tendon • Surgically induced damage	***Treatment*** • MMP 9 inhibitor • Daily administration of MMPI starting @ the day of surgery, until day 8 post surgery ***Analysis***: • 7-35 days post surgery	***Biomechanical*** • No difference in biomechanical properties (stiffness, and max load at failure) ***Histological*** • No alteration in tendon morphology ***Biochemical*** • Decreased MMP9 activity
Pasternal et al, [79] 2006	• *In vivo* • Rat • Achilles • Surgically induced damage	***Treatment*** • Doxycucline • Starting @ 1 day before surgery until the time of sacrifice ***Analysis***: • 5, 8, and 14 days after surgery	***Biomechanical*** • Decreased force at failure, and energy uptake ***Histological*** • None ***Biochemical*** • None

It can be concluded from Table 2.2 that using broad-spectrum MMPIs (BI; e.g., doxycycline and alpha-2-macroglobulin) in surgically induced tendinopathy models results in greater collagen organization[14,25,44,70], as well as a reduction in collagen degradation[14,70], limited MMP activity[25,44,70], and increased mechanical strength[25,44,70].

The effect of MMPIs on tendons in clinical trials

Beside laboratories, MMPIs have also been used in clinical trials to improve tendon healing. Aprotinin is a broad-spectrum MMPI which was initially used for limiting blood loss during surgery and promoting soft tissue healing after surgery[73]. Years later, it was introduced as therapy option for chronic tendinopathy. In clinic, aprotinin has been used for treating chronic patellar and Achilles tendinopathies. In one randomized control trial, aprotinin presented superior results than both corticosteroid and saline injections in chronic patellar tendinopathy, 12 months after injection[17]. The results are mixed about the effect of aprotinin on Achilles tendinopathy. Some uncontrolled or poorly controlled studies reported high rates of success in management of chronic Achilles tendinopathy with aprotinin[9,19]. In a study by Brown *et al*[17] on chronic tendinopathy, there was no significant difference but a trend toward better results by aprotinin than placebo. Another clinical outcome[73], demonstrated that in treating chronic tendinopathy, using aprotinin led to more successful results for treating mid-Achilles tendons than patellar tendons.

Although excess activity of MMPs may lead to degeneration and weakening of ECM, normal actions of MMPs are needed for ECM remodeling and repair. It should be noted that applying MMPIs to promote healing in some cases may adversely affect the healing process. In this regard, Pasternak *et al*[79] conducted a test evaluating the effect of broad-spectrum MMPI, doxycycline, in the prevention or healing of the acute injury of Achilles tendon in rats *in vivo*. Their results demonstrated negative influence of doxycycline on early stages of tendon healing as measured in tension test by force and energy uptake at failure. These results could be explained by the fact that there was tendon regeneration at the site of injury consisting of a loose collagenous network including mostly proteoglycan and collagen III. MMPs are fundamental to remove this newly formed loose callus to be replaced by more densely organized collagens, mostly collagen I. In fact, this removal is

needed for remodeling and consequently building stronger tissue. The negative result from this study and positive result from literature reviewed in the previous section imply that the effect of MMPIs on tendon healing is strongly case-dependent.

Therefore, before applying MMPIs to prevent or heal tendinopathy, this dual activity of MMPs should be taken into account. That is, while in one pathologic model, specific MMP might play a negative role, in another pathologic model, the role of that specific MMP might be positive on tissue quality. In conclusion, the alteration in the presence and activity of MMPs should be studied in each pathologic model, to be able to target MMPs which are destructive to tendon health.

Narrow or broad-spectrum MMPIs

There are some evidence that broad-spectrum MMP inhibition impairs tendon healing, since MMPs play fundamental role in remodeling and healing[46,54,75,79]. Therefore, more selective inhibition might lead to positive effects. In fact, it would be beneficial to target specific MMPs which are detrimental to tendon healing, without affecting MMPs which act during tendon healing. This could be achieved by applying narrow-spectrum MMPIs (NI) instead of broad-spectrum MMPIs (BI).

Obviously, choosing appropriate NI, requires identifying MMPs deleterious to tendon healing. Based on the information in the literature regarding destructive MMPs present in tendinopathic rat tendons, MMPs 9 and 13 have first been selected as target MMPs to inhibit by our group. It should be noted that RT-PCR analysis has been performed by our group on 6-day stress-deprived RTTs and an increase in MMP9 and MMP13 has been reported (Figure 2.2). However, for our *ex vivo* experiment, we focused on MMP13. It is because MMP13 in rats is equivalent to MMP1 (interstitial collagenase) in human and because its expression has been reported to be increased in pathological human tendons[5].

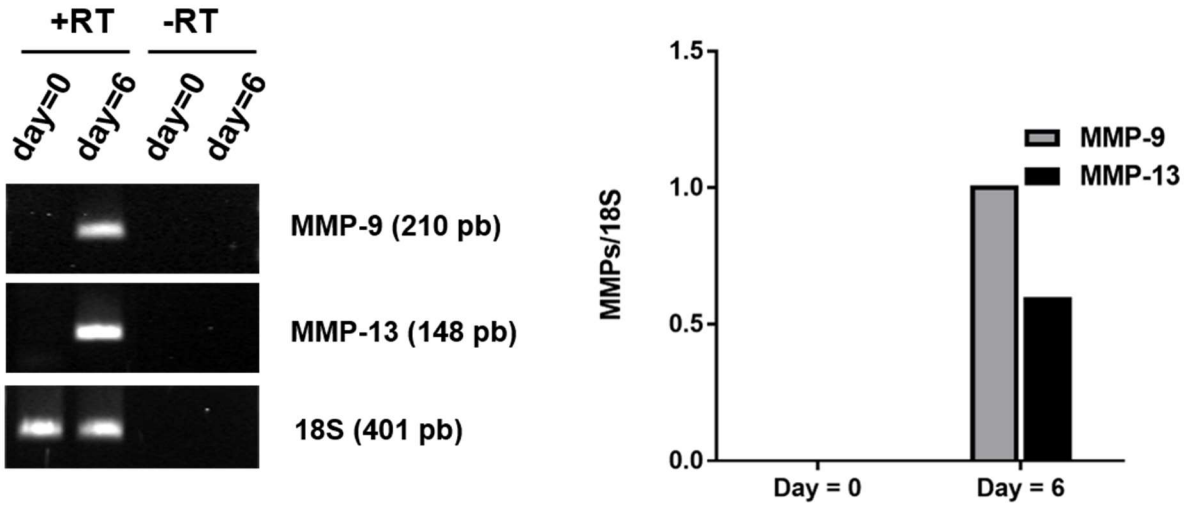

Figure 2.2 Increased MMP9 and MMP13 in 6-day SD tendon samples by PCR analysis.

2.3.2. PRP: general information

PRP therapy is one new alternative in tendon healing. PRP is a blood product obtained from whole blood that contains a high concentration of platelets. The beneficial effect of PRP is probably related to providing an environment full of bioactive molecules and GFs appropriate for tissue regeneration. This environment enables cell migration and proliferation, angiogenesis, and matrix deposition in tendon healing[11].

Clinical use of PRP in tendon healing has been expanded, despite the lack of enough randomized control trials with large scales[11]. However, in order to clinically use PRP safely and more efficiently, more high-quality *in vivo* and *ex vivo* studies should be conducted.

In the following section, the PRP biological activity will be explained with more details. Then the current state-of-the-art in using PRP in tendon healing in *ex vivo* and *in vivo* animal studies, and in clinical trials will be introduced.

PRP biological activity

The beneficial effects of PRP at molecular levels is attributed to the high concentrate content of GFs and proteins of cytokine and chemokine families which are secreted from activated platelets[105]. Platelets can be activated, i.e. aggregated, endogenously during injury to the vessel wall, or exogenously by being exposed to bovine thrombin, calcium, or collagen type 1[105]. Releasing GFs and proteins to the site of injury improves tissue regeneration. Some GFs identified in PRP include platelet-derived growth factor (PDGF), transforming growth factor (TGF) β, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Table 2.3 demonstrate the roles of these growth factors produced by activated platelets[94].

Table 2.3 GFs released by activated platelets[86].

GF	Function
TGF-β1	Matrix synthesis
PDGF	Stimulate angiogenesis, cell proliferation, mitogen for fibroblasts
bFGF	Proliferation of fibroblasts and myoblasts, angiogenesis
VEGF	Angiogenesis
EGF	Proliferation of epithelial and mesenchymal cells
IGF-1	Stimulate fibroblasts and myoblasts
HGF	Angiogenesis

bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

The effect of PRP on tendons in *ex vivo* and *in vivo* animal studies

PRP enhances tendon healing by promoting tissue regeneration. The rationale behind using PRP is to have concentrated platelets at the site of tendon injury. These activated, more concentrated platelets release GFs which benefit tissue healing. PRP affects tissue in several aspects:

GFs: One of these aspects is increasing GFs such as PDGF and TGF, and VEGF. Increasing PDGF and TGF enhance recruitment of inflammatory cells, which consequently release additional growth factors[11,68,89]. Moreover, VEGF which plays a key role in signaling angiogenesis pathway is affected by PRPs. In fact, PRP enhances vascularisation, and consequently tendon healing of surgically induced tendinopathies, *in vivo* by increasing VEGF expression[11].

Tenocyte proliferation: *In vitro* application of PRP on healthy tendons significantly increases tenocyte proliferation[3,68]. However, the positive or negative clinically result of tenocyte proliferation has not yet been investigated[11].

Collagen expression: PRP affects collagen expression by increasing total collagen expression. De Mos *et al*.[68] reported a decrease in the gene expression of both collagen I and collagen III of healthy tendons treated with PRP, while increasing the overall collagen production. This increase in total collagen production is probably attributable to cell proliferation. However, they reported no change in the ratio of Collagen I / Collagen III. In another study, there was a significant increase in mRNA expression of collagen I, collagen III, and ratio of collagen I / collagen III[89]. More studies are needed to clarify the effect of PRP on collagen I and collagen III expression.

Rate of repair: Some *in vivo* animal studies showed that PRP-treated tendons, with surgically induced tendinopathy, healed faster than control tendons[56–59]. In fact, they reported enhanced vascularisation in the PRP-treated group leading to accelerated healing process.

Quality of repaired tendon

Histology: Using PRP enhances the histological quality of repaired tendon. More mature, dense and better organized collagen fibers, with more oriented tenocytes have been reported in some *in vivo* animal studies surgically induced tendinopathy[16,56–59].

Strength: PRP enhances the mechanical properties of regenerated tendons which were exposed to surgically induced tendinopathy. Lyras *et al*, reported an increase in force at

failure, ultimate stress, and stiffness of the PRP-treated group compared with control group[58]. In another study, the repaired tendon in PRP-group featured higher strength at failure and elastic modulus[16].

MMP expression: PRP alters gene expression of matrix metalloproteinases (MMPs). In human tenocyte culture, PRP increases MMP1 and MMP3 slightly. However, MMP13 expression was not affected by PRP[68]. In another *ex vivo* study on equine flexor digitorum superficialis tendon, no change was reported in MMP3 and MMP13 gene expression in response to PRP treatment[89]. It should be noted that with the current knowledge, it is difficult to state whether increasing MMP1 and MMP3 expression is beneficial for tendon repair[68].

Altogether, based on the literature, there are some evidences of the beneficial effect of using PRP to heal **traumatic** tendon injury. However more high-quality studies are needed to assess biological activity of PRP and its effect on tissue. Moreover, whether these results are applicable to **CT** is to be elucidated. In the next section, the results of using PRP to heal CT is presented.

The effect of PRP on tendons in clinical trials

Using autologous blood product such as PRP seems a natural and safe way to improve healing. Clinical trials have shown promising results, however more high-quality clinical trials with long-term follow-up are needed to prove the efficacy of PRP in tendon disorders healing[94]. The efficacy of PRP in healing of tendinopathies such as lateral elbow tendinopathy, rotator cuff repair, Achilles tendon repair, and patellar tendinopathy has been investigated in clinical trials. The results are summarized bellow.

Elbow tendinopathy: In a retrospective study by Mautner *et al*[63], patients with different chronic tendinopathies including elbow tendinopathy, received PRP injection. Ninety three percent of patients with elbow tendinopathy reported moderate improvement to complete resolution of symptoms. In a randomized control trial[81], application of PRP versus corticosteroid resulted in a significant increase in functionality and reduction of pain of tendons with CT. In another control study, patients with chronic elbow tendinosis

receiving PRP demonstrated improvement in their visual analog pain scores to 60%, 81% and 93% at 8 weeks, 6 months, and 25.6 month post-treatment, respectively[66]. A review study mentions that currently there is more evidence for the efficiency of PRP in treating lateral elbow tendinopathy than other anatomical areas[94].

Rotator cuff tendinopathy: In Mautner *et al* study[63], 81% of patients with rotator cuff CT reported moderate improvement to complete resolution of symptoms at least 6 month after receiving PRP injection. There are also some studies investigating the effect of PRP on rotator cuff acute injury.

Achilles tendinopathy: In Mautner *et al* study[63], 100% of patients with chronic Achilles tendinopathy reported moderate improvement to complete resolution of symptoms at least 6 month after receiving PRP injection. However, in a randomized control trial study, PRP injection in patients with chronic midportion Achilles tendinopathy combined with an eccentric training program revealed no benefit in clinical and structural properties of tendon over placebo injection at 1 year follow-up[41]. There is a need for randomized control trials to clarify the effect of PRP therapy on Achilles tendon disorder.

Patellar tendinopathy: In Mautner *et al* study[63], 59% of patients with chronic patellar tendinopathy reported moderate improvement to complete resolution of symptoms at least 6 month after receiving PRP injection. In a small non-control study, 20 patients with chronic patellar tendinopathy received PRP injection. There was encouraging results with regard to reducing pain and regaining functionality at 6-month follow-up[47]. Large-scale controlled studies with longer follow-up are needed on this subject.

Altogether, conducting further preclinical models are essential to define tendinopathy features and verify the efficacy of treatments. Moreover, it seems that part of treatment strategies should focus on combination of treatments in order to benefit from their strengths and control their weakness.

2.4. Problematic

Tendon is a soft tissue which transmits the force generated by muscle to bone. Tendon injury causes marked morbidity and may have a significant impact on many aspects of the patients' life including work, sport, and even daily needs. Unfortunately, tendon injuries are highly prevalent. By increasing the number of people participating in physical and recreational activities every year, more people will likely be involved with soft tissue disorders resulting in increasing health care cost, and patient morbidity[40]. Even people with no intensive activities are prone to tendinopathy. For example, in a study of 58 patients with Achilles tendinopathy, 31% of whom had no vigorous physical activity[84].

Although tendon disorder is a common and growing problem, the ideal treatment is still unclear. Traditional treatments may not be the most effective option because they have a high emphasis on anti-inflammatory strategies, while in most cases the pathology underlying tendinopathy is degeneration and not inflammation. There are numerous newer treatments currently available to cure tendinopathy. However, many of these treatments have a poor or no evidence base[84].

Defining the aetiology and pathogenesis of tendinopathy may lead to more rational and efficient treatments. The aetiology of tendinopathy seems to be multifactorial, and the pathogenesis of tendinopathy is still unclear[101]. While there has been some progress in understanding tendinopathy, the condition remains poorly understood[27,111]. Moreover, studying the pathogenesis of tendinopathy in human is difficult, because the available tendinopathy samples come from individuals with advanced pathology[27]. Therefore, there is a need to develop animal tendinopathy models, *ex vivo* or *in vivo*, to better understand tendinopathy and develop treatments for this disorder. While animal models have been frequently used to study tendinopathy, most of these studies have investigated either early tendinopathy or advanced tendinopathy, and the transient mid-term tendinopathy has been given less attention. We refer to this transient stage between early and advanced tendinopathy, as moderate tendinopathy. Moderate-stage tendinopathy becomes even more important when investigating the potential efficacy of treatments. This is because early-stage human tendinopathy is often asymptomatic[48,65] and patients often do not seek

medical attention. On the other hand, advanced tendinopathy usually requires invasive surgery for treatment[51]. Therefore, moderate tendinopathy would be a better time for studies that aim to estimate the effectiveness of new pharmacotherapies.

To the best of our knowledge, there is no clear clinical translation of different levels of tendinopathy. However, we suggest moderate hiatopathological features in the tendinopathic tendons could probably represent a moderate tendinopathy in clinics. That is, for example, hypercellularity; several partial collagen tears without a complete rupture; changes in the intensity of imaging signals which are typical of well-aligned collagen fibers, at ultrasonography (US) or magnetic resonance imaging (MRI).

This research study aims at creating moderate tendinopathy model *ex vivo* for the study the pathogenesis of tendinopathy and to investigate the efficacy of promising and new treatments, consisting of selective MMPIs and PRP, applied either alone or in combination.

2.5. Research hypotheses

The hypotheses of this research study are:

1. A rat tail tendon develops moderate tendinopathy after 4 days of stress deprivation in culture conditions *ex vivo*;
2. The combination of NI and PRP is more efficient for the treatment of moderate tendinopathy *ex vivo* than each modality alone.

2.6. Objectives

The objectives of this project are:

1. Characterizing moderate tendinopathy model *ex vivo*.
    a. Characterizing rat tail tendinopathy model via stress deprivation *ex vivo*.
    b. Verifying the validity of the model by comparing the achieved results in terms of histological, biochemical, and mechanical features with control tendons and tendinopathy features documented in literature.

2. Studying the effect of two treatment modalities (MMP inhibitor and PRP injection), in combination and alone, on the *ex vivo* tendinopathy model.
    a. Applying MMP inhibitor and PRP, in combination and alone, on created model of moderate tendinopathy *ex vivo*.
    b. Evaluating the efficacy of treatments by comparing the changes in histological, biochemical, and mechanical features of treated tendinopathic tissues, untreated tendinopathic tissues, and fresh healthy tendons.

Chapter 3. Characterization of moderate tendinopathy in *ex vivo* stress-deprived rat tail tendons

3.1. ABSTRACT

Stress deprivation (SD) has frequently been used as a model to study tendinopathy. Most of these studies have investigated either short-term (early tendinopathy) or long-term SD (advanced tendinopathy), while the transient mid-term SD has been given less attention. Therefore, the main objective of this study was to characterize mid-term SD. To this end, live, healthy rat tail tendons (RTTs) were harvested and cultured without mechanical stress and then were divided into five groups based on their culture time (fresh, 2-day SD, 4-day SD, 6-day SD, and 10-day SD). For each group, the tendons were subjected to traction testing and histopathological, biochemical, and viability assays. Our results showed that 4 days of SD resulted in moderate pathological changes in RTTs. These changes included increases in the space between fibers, cell density, and collagen tortuosity as well as a decrease in collagen density and elongation of cell nuclei. No changes in the stress at failure of tendons were observed at this time point. This simple *ex vivo* model is expected to be useful for studying the progression of tendinopathy as well as for testing potential mechanobiological or pharmacological strategies to stop or reverse the progression of the pathology.

Keywords: animal, stress deprivation, tendon, mechanical properties, biochemical properties, histology, degradation, MMP, collagen

3.2. INTRODUCTION

Tendinopathy is a frequent health problem that accounts for over 65% of work-related musculoskeletal disorders[31]. It is important not only to study and understand tendinopathy because of its prevalence but also to find an optimal treatment for the

disease. Many treatment modalities have been proposed for tendinopathy (e.g., nonsteroidal anti-inflammatory drugs, stem cell or gene therapy, eccentric exercises, laser therapy). However, there is very little support of the efficacy of these treatments[106]. In vitro, *ex vivo* and *in vivo* models of tendinopathy are part of a multiscale approach to understand and develop treatments for tendon disorders[15,48]. Stress deprivation (SD) of tendon tissues has been frequently used to develop such models[6,30,34]. SD can be categorized as short term or long term based on the investigation time points and the obtained results. For example, studies using short-term *ex vivo* SD models at 24 h, 48 h, and 72 h[6,30,34] observed increases in apoptosis, cell roundness, and MMP 13 gene/protein expression as well as a decrease in fiber density. In addition to these early changes, long-term studies at 6 days, 12 days[102], 1 week, 2 weeks, and 8 weeks[1,5,37,49] observed increases in collagen fiber waviness and disorientation, a decrease in mechanical properties and an increase followed by a decrease in cell density. Table 3.1 summarizes the results from previous *ex vivo* studies on stress-deprived tendons. These studies allowed characterization of short-term (early) and long-term (advanced) tendinopathy models. However, there is still a need to study different time points within this interval to better understand the transition of the tissue condition during the progression from early to advanced tendinopathy. This transition state shall be referred to as moderate tendinopathy.

In addition to studying early and advanced tendinopathy, studying moderate tendinopathy is crucial to understand all stages of damage that the tendons experience during lesion development. Moderate-stage tendinopathy becomes even more important when investigating the potential efficacy of treatments because early-stage human tendinopathy is often asymptomatic[48,65] and patients often do not seek medical attention. Advanced tendinopathy, however, usually requires invasive surgery for treatment[51]. Therefore, moderate tendinopathy would be a better time for studies that are designed to estimate the effectiveness of new pharmacotherapies.

Therefore, in the present study, we designed a moderate SD model consisting of a 10-day SD experiment with measurements taken every 2 days until day 6, and then at day 10 for the histological, biochemical, and mechanical end-point assays.

Table 3.1 Data extracted from *ex vivo* SD studies. N/A stands for data not available.

Reference	**Model**	**Culture Duration**	**Results**
Short-term SD (early tendinopathy)			
Egerbacher *et al*,[29] 2008	Rat, Adult, Tail tendon	24 hours	**Biomechanical** • None **Histological** • Increase in cell apoptosis **Biochemical** • None
Egerbacher *et al*,[30] 2006	Rat, Adult, Tail tendon	24 hours	**Biomechanical** • None **Histological** • Decrease in collagen fiber density

			Biochemical • Increase in MMP13 expression and MMP13 protein synthesis
Arnoczky, S. P. *et al*,[6] 2008	Rat, Adult, Tail tendon	1,2,3 days	**Biomechanical** • No change in maximum stress **Histological** • Cell roundness **Biochemical** • Increase in MMP13 expression
Gardner *et al*,[34] 2008	Rat, 6 months old, Tail tendon	24, 48, 72 hours	**Biomechanical** • None **Histological** • None **Biochemical** • Increase in MMP13 expression

Long term-SD (advanced tendinopathy)			
Wang *et al*,[102] 2015	Rabbit, Female, 15 weeks old, Achilles tendon	6,12 days	**Biomechanical** • None **Histological** • Increase in cell apoptosis • *Day 6*: Increase in cell density; *Day 12*: Decrease in cell density • Cell roundness • Loose collagen fibers • Increase in space between collagen fibers • Wavy and disoriented fibers **Biochemical** • None
Arnoczky *et al*,[5] 2007	Rat, Adult, Tail tendon	7 days	**Biomechanical** • Decrease in ultimate stress, tensile modulus and strain at ultimate stress **Histological** • Less dense collagen fiber packing

			Biochemical • Increase in MMP13 expression and MMP13 protein synthesis
Abreu *et al*,[1] 2007	Rat, Male, Adult, Tail tendon	1 week	**Biomechanical** • Decrease in elastic modulus **Histological** • None **Biochemical** • Decrease in GAG* content
Lavagnino *et al*,[49] 2005	Rat, 13 months old, Tail tendon	21 days	**Biomechanical** • None **Histological** • No change in collagen fiber density **Biochemical** • None

Hannafin *et al*,[37] 1995	Canine, Adult, mixed age and sex, Flexor digitorum tendon	8 weeks	**Biomechanical** • Decrease in tensile modulus **Histological** • Decrease in cellularity • Cell roundness • Mild increase in collagen crimps **Biochemical** • None

*: Glycosaminoglycans: an ECM constituent.

3.3. MATERIALS AND METHODS:

Animals. Experiments were carried out on male Sprague Dawley rats weighing 500-800 g (4 to 6 months old) (Charles River; St. Constant, Québec, Canada). Animal housing and the experimental protocol (protocol #EL2013-01) were carried out in accordance with regulations set by the Canadian Council of Animal Care Committee and were approved by the Animal Care Committee of the University of Sherbrooke (CIPA/CFPA-FMSS).

RTT extraction. Tendon isolation and preparation were conducted as described in a previous study[18]. The distribution of rats and tendons for mechanical testings and viability, histology and biochemical analysis are shown in Figure 3.1. All manipulations were performed in a cold Dulbecco's phosphate buffered saline (D-PBS) solution (311-410-CL; Wisent Inc., St-Bruno, Canada) containing 1 g/L glucose (609-037-EL; Wisent Inc.) and 1% antibiotic–antimycotic (15240-062; Invitrogen, Burlington, Canada). Following isolation, tendon cross-sectional areas were evaluated using an optic micrometer and a stereomicroscope[76]. Briefly, the tendons were maintained in the saline solution to avoid dehydration and rotated along their longitudinal axis. Images of specimen projections were captured at 10° angular increments and the tendon edges were localized within a local reference frame using a contrast-based image analysis algorithm. The cross-sectional areas were estimated using a profile reconstruction algorithm. The tendons were then washed 5 times under sterile conditions. Afterwards, they were transferred to tissue culture flasks containing DMEM solution (12800-017; Invitrogen, Burlington, Canada) supplemented with 3.7 g/L of sodium bicarbonate (600-105-CG; Wisent Inc.), 10% FBS (090150; Wisent Inc.), and 1% antibiotic–antimycotic.

RTT incubation. Tendons were divided into five groups in the core experiments and three groups in the secondary experiments based on culture time (Figure 3.1). In each experiment, fresh tendons were used as controls to the stress-deprived (SD) tendons. SD tendons were incubated under tissue culture conditions (37 °C, 95% humidity, 5% CO2, sterile environment) for 2, 4, 6, or 10 days. In the core experiments, there were three tendons in each group, two for mechanical testing and one for the histology and biochemical studies. In these experiments, the mechanical results from the two tendons

were averaged for each group. In the secondary experiment, there were two tendons per group, one for mechanical characterization and one for the histology and biochemical studies. The media were changed every 2 days.

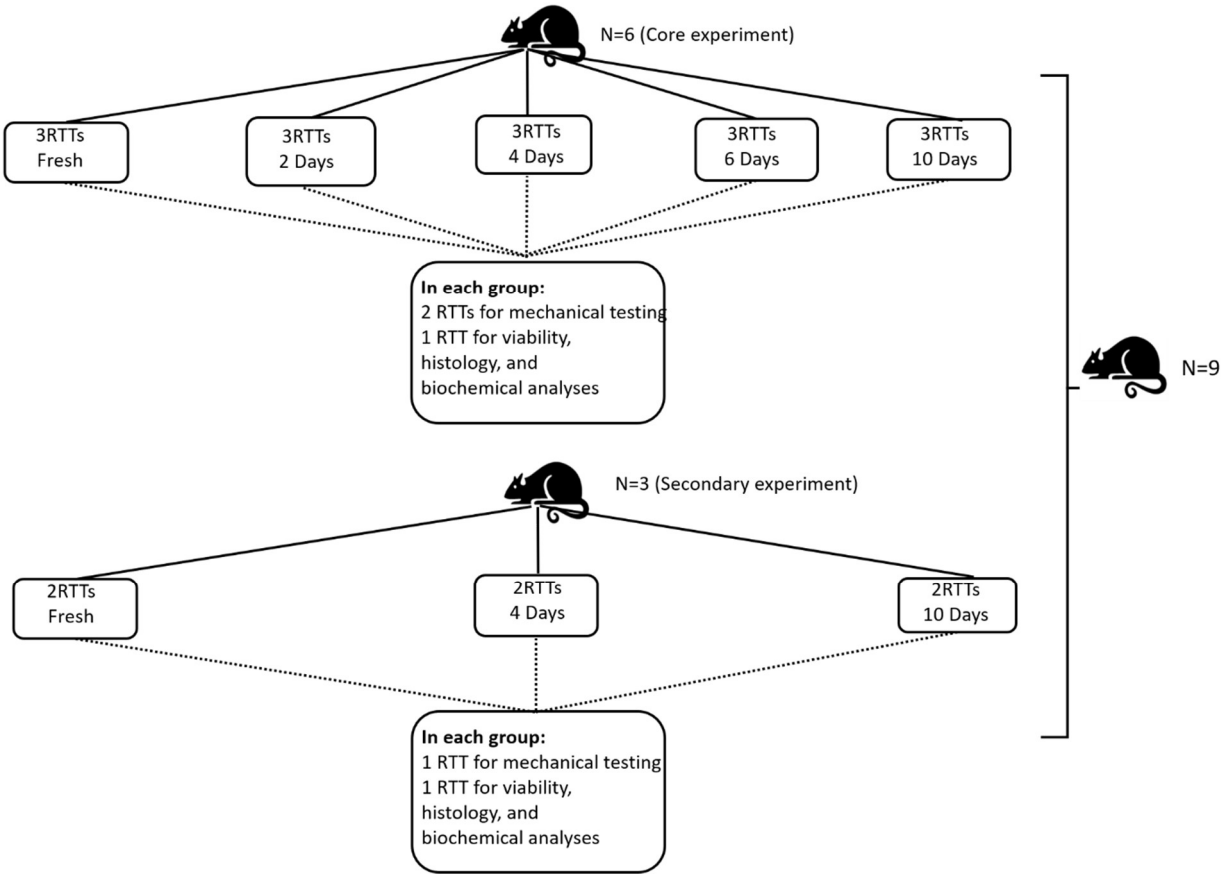

Figure 3.1 Number and distributions of tendons from each rat. RTT stands for rat tail tendon, and 2 Days, 4 Days, 6 Days, and 10 Days stand for 2, 4, 6, and 10 days of stress deprivation, respectively. In the core experiment, there were three tendons in each group, two for mechanical analysis, and one for viability, histology, and biochemical analyses. In the secondary experiment, there were two tendons per group: one for mechanical analysis and the other for viability, histology, and biochemical analyses.

RTT mechanical characterization. Mechanical tests were conducted on live tissues at the end of their culture time (i.e., 0 (control), 2, 4, 6, or 10 days). At the time of mechanical testing, the ends of the tendon were wound around cylinder-shaped anchors and allowed to dry briefly on the top face of the anchors. A small drop of ethyl cyanoacrylate (10300;

Krazy Glue, Columbus, OH) was applied to the portion of the tendon at the top of the anchor. The tendons were then transferred to a custom-manufactured bioreactor[77]. The specimens were subjected to the following testing protocol: The initial zero-strain reference was defined by achieving a tension load of 3 g at equilibrium. Preconditioning was performed with a series of 120 sinusoidal waves at two different amplitudes (60 cycles at 1% strain; 60 cycles at 2% strain) at 1 Hz. The final zero-strain reference was defined by again reaching a tension load of 3 g at equilibrium. Afterwards, the tendons were subjected to a traction test (at a strain rate of 0.5%/s). Maximum engineering stress before failure was used as a measure of tendon strength. Data are reported as normalized to day 0.

Viability test. Viability tests were conducted on RT tissues at the end of their culture time (i.e., 0, 2, 4, 6, or 10 days). Viability of cells from the fresh and cultured tendons was assessed using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (L3224; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Tendons were washed in D-PBS to remove any serum and then incubated with ethidium homodimer-1 (1 μM for 45 min) followed by treatment with calcein AM (1 μM for 40 min) in D-PBS at room temperature. The tendons were washed briefly in D-PBS prior to being mounted on glass slides. Green-fluorescing live cells and red-fluorescing dead cells were visualized within 45 min with an EVOS FL Auto microscope (Thermo Fisher Scientific, Massachusetts, USA). Images were captured with a digital camera.

Immunofluorescence staining of MMP-13 protein. We examined MMP13 because it is equivalent to MMP1 (interstitial collagenase) in human and because its expression has been reported to be increased in pathological human tendons[5]. Immunohistochemical studies of MMP-13 were conducted on tissue samples that had been previously fixed in 10% neutral buffered formalin (032-060, Fisher Scientific, Company LLC, Kalamazoo, MI) and embedded in the optimal cutting temperature (OCT)-embedding medium. Embedded tissues were longitudinally cut into 20 μm sections using a cryostat (Thermo Scientific, Cheshire, UK). Sections were incubated in 0.1 M glycine (56-40-6, Roche diagnostic GmbH, Mannheim, Germany)/D-PBS for 10 min to wash away the mounting medium used in the

tissue sectioning. Sections were incubated in D-PBS/0.1% Triton (T8787, Sigma Aldrich, Merck KGaA, Darmstadt, Germany)/2% bovine serum albumin (BSA) (A7030, Sigma Aldrich, Merck KGaA, Darmstadt, Germany) at RT for 30 min inside a humid chamber. They were then washed once in 0.1 M glycine/D-PBS and five times in D-PBS. After washing, sections were incubated with the rabbit polyclonal anti-MMP13 antibody (ab39012, Abcam, Cambridge, UK, 20 µg/µl; all antibodies are diluted in D-PBS/0.05% Triton/1% BSA) and DAPI (62248, Sigma Aldrich, Oakville, Canada, 1 µg/mL) at room temperature for 1 h. Sections were washed five times in D-PBS and were incubated with the secondary antibody (A11034, Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies, Carlsbad, California, United States; 20 µg/µl) at room temperature for 1 hour. They were washed in D-PBS, mounted on microscopy slides with mounting media (F4680, Sigma Aldrich, Oakville, Ontario, Canada) and sealed with nail polish. Negative controls included sections incubated with the rabbit isotype IgG antibody, (X0903, Agilent Dako, Santa Clara, US; 20 µg/µl, diluted in D-PBS/0.05% Triton/ 1% BSA) without being exposed to the primary antibody. Green (MMP-13 proteins) and blue (nucleus) fluorescence images were captured with an EVOS FL Auto microscope (Thermo Fisher Scientific, Massachusetts, USA).

The 20X images of MMP-13 protein were analyzed using Image-Pro Plus software (version 6.0.) to quantify the intensity of MMP-13. Three ROIs focusing on the central zones of RT were captured from each image. The MMP-13 intensity was calculated as the sum of the density of signals in each pixel from each ROI. The results from the three ROIs were averaged. Data are reported as normalized to day 0.

RT-PCR analysis of MMP-13 mRNA transcript expression

In preliminary experiments, total RNA extraction from frozen tissues was performed using the TRIspin method[85], which combines the Trizol method with the RNeasy Total RNA Kit (Qiagen). RNA integrity was assessed with an Agilent RNA 6000 Nano LabChip according to the manufacturer's protocol. Two micrograms of RNA from each sample was converted to cDNA using the Superscript First-strand RT-PCR Kit (Invitrogen) with random primers that were then subjected to PCR amplification using the Platimun Pfx

Polymerase Kit (Invitrogen). To ensure the absence of genomic DNA contamination, RT reactions were also conducted in the absence of reverse transcriptase (-RT). Comparative quantifications of MMP-13 expression levels at each time point were performed by quantifying their respective PCR products using an Agilent DNA 1000 Labchip and expressed as ratios of the amount of each MMP amplicon over that of the corresponding 18S[88].

Histological characterization. Histological studies were conducted on RT tissues fixed in 10% neutral buffered formalin and embedded in either paraffin for hematoxylin and eosin staining or in OCT medium.

Paraffin-embedded tissues were longitudinally sectioned into 6 μm-thick sections and were processed in hematoxylin and eosin (H&E) stain for light microscopy. Images of each sample were captured using whole slide imaging with a Nanozoomer (Hamamatsu Photonics K.K., Hamamatsu city, Japan) in visible mode. The 20X images were analyzed using the following five parameters: fiber density, areas of spaces between fibers, fiber tortuosity, tenocyte density and nucleus shape.

The two first parameters of the extracellular matrix were evaluated using Image-Pro Plus software as described previously[38]. For each micrograph, three regions of interest (ROIs) were captured taking care not to include damages caused by the cutting blade. The ROIs were then analyzed with Image-Pro Plus software. Data are presented as normalized to day 0.

Fiber density. The background density was found by contrast and separated into two categories: background (red) and fibers (black). The fiber density was calculated as:

$$Fiber\ density\ (\%) = \left(1 - \frac{Number\ of\ pixels\ of\ background}{Number\ of\ pixels\ of\ ROI}\right) \times 100\ \% \qquad (3.1)$$

Results from the three ROIs were averaged.

Areas of spaces between fibers. For each ROI, the areas of the spaces between fibers were also evaluated in terms of pixel numbers and then averaged. Data are reported as normalized to day 0.

Fiber tortuosity. For collagen fiber tortuosity analysis, digital images of H&E stained slides were randomly numbered for blind analysis. Fiber tortuosity was scored between 0 and 3 using a semiquantitative scale similar to the Bonar scoring method6. 0 was assigned to images that showed the least collagen tortuosity (collagen fibers were mostly aligned in the longitudinal direction of tendon), whereas 3 was assigned to images with the waviest collagen fibers. Data are reported as normalized to day 0.

Tenocyte density. Tenocyte density was evaluated as:

$$Tenocyte\ density = \frac{Number\ of\ tenocytes}{Tendo\quad longitudinal\ area} \tag{3.2}$$

Using a grid with a known scale bar, the number of cells was counted, and the tendon longitudinal area was calculated. Data are reported as normalized to day 0.

Nucleus shape. Longitudinal cryo-sections of formalin-fixed tissues of 20 μm thickness were stained with DAPI (DAPI, Sigma Aldrich, 62248, 1 μg/mL). Images of blue nuclei were captured using whole slide imaging with the Nanozoomer. The 40X images were analyzed using Image-Pro Plus software. The nucleus shape was recognized by contrast, and its roundness was evaluated as:

$$Nucleus\ roundness = \frac{Perimeter\ of\ nucleus^2}{4 \times \pi \times Area\ of\ nucleus} \tag{3.3}$$

A value of 1 indicates a perfect circle, whereas higher values indicate more elongated shapes. Data are reported as normalized to day 0.

Statistical analyses

Data are represented as the means ± standard error of mean (SEM). Mechanical, histological, and biochemical results were compared using nonparametric Wilcoxon

matched-pairs signed rank tests (GraphPad prism, 7.03) or one-median Wilcoxon tests (IBM SPSS) comparing groups to day 0. Significance was set at p values < 0.05.

3.4. RESULTS

Cross-sectional area

The cross-sectional areas of tendons were evaluated to 0.036 ± 0.002 mm^2.

Cell Viability

Under fluorescence microscopy, control (day 0) samples showed a high number of green cells and a few red cells, indicating a high level of viability (Figure 3.2a). As the SD time increased, samples revealed a gradual increase in the number of red cells (Figure 3.2b, c, d, e). Qualitative observations suggest that there was an increase in the rate of cell death after 6 and 10 days of SD compared to that at earlier time points (Figure 3.2d, e).

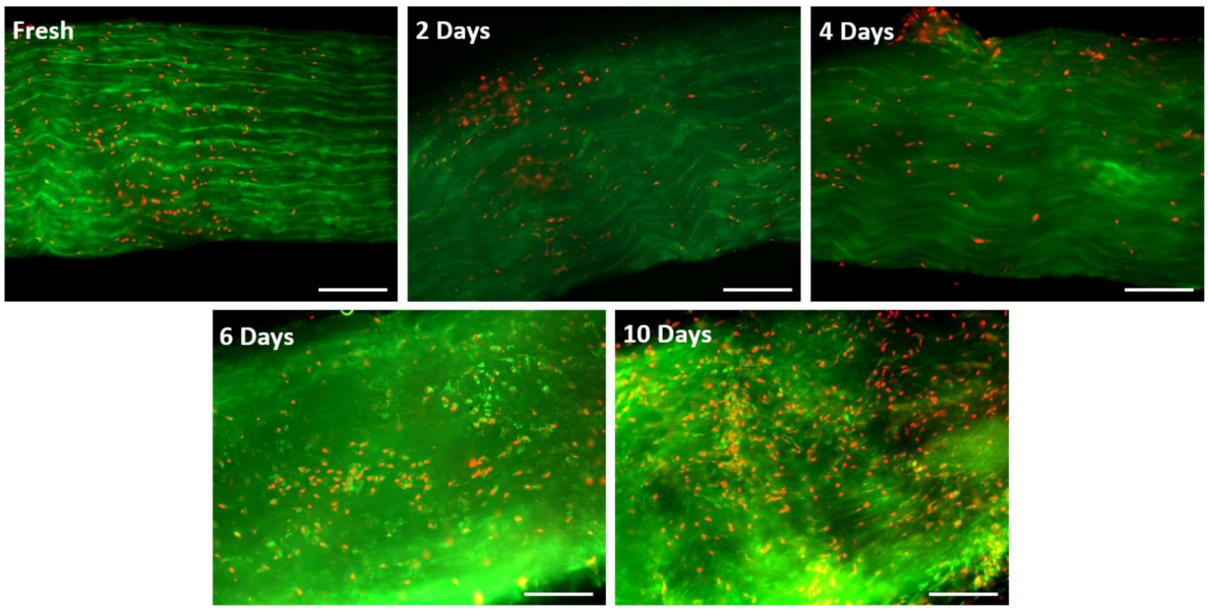

Figure 3.2 Typical fluorescence micrographs obtained from freshly isolated tendons experiencing 2, 4, 6, and 10 days of stress deprivation. Green-fluorescing cells are alive, and red-fluorescing cells are dead. Bar = 200 µm.

Mechanical results

The stress at failure of fresh tendons was evaluated to 5.3 ± 1.0 MPa. The effect of SD on the maximum stress at failure of the RTTs is demonstrated in Figure 3.3. There were no changes in the maximum stress at failure after 2 and 4 days of SD compared to that for the

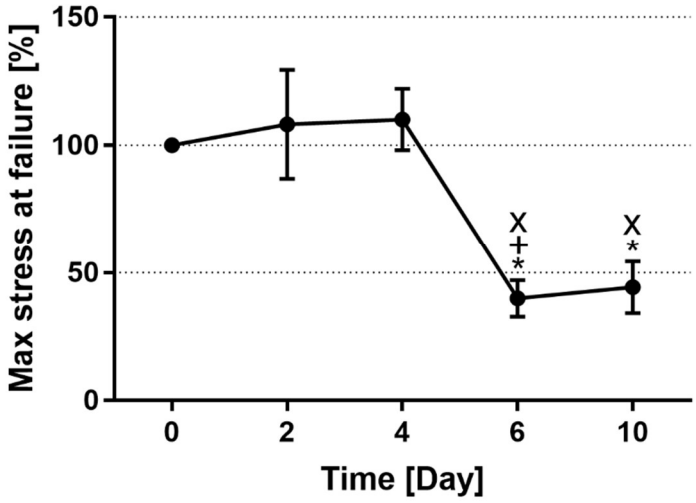

Figure 3.3 Changes in maximum stress at failure (mean and SEM) with stress deprivation. * indicates a significant difference ($p<0.05$) from day 0 (fresh tendons); +, a significant difference ($p<0.05$) from 2 days of stress deprivation; and x, a significant difference ($p<0.05$) from 4 days of stress deprivation.

day 0 control. However, at 6 and 10 days of SD, there was a statistically significant decrease in the maximum stress at failure to approximately 40% of the initial stress at day 0.

Histology

Under light microscopy, freshly harvested samples showed dense and well-aligned collagen fibers (Figure 3.4a). Fiber density and space area between fibers of fresh tendons were evaluated to 92.4 ± 0.9% and 42.5 ± 3.5 pixels, respectively. Following SD, tendons exhibited more loosely packed and wavy collagen fibers and had increased space between collagen fibers compared to the fresh control samples (Figure 3.4b-e). Moreover, under SD conditions, tendon cells started to lose their elongated shape and became more

rounded in shape (Figure 3.4b-e).

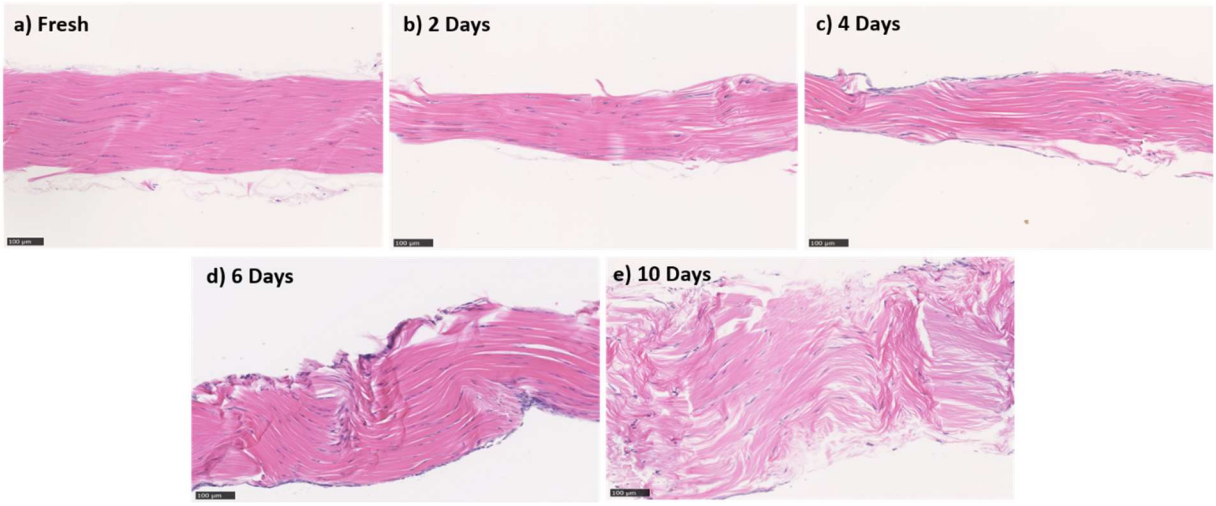

Figure 3.4 Typical micrographs of longitudinal sections of tendons stained with hematoxylin and eosin. Bar = 100μm.

The roundness of the nuclei of fresh tendons was evaluated to 2.3 ± 0.1. Quantitative analyses confirmed our qualitative observations regarding the space between fibers, fiber density, and nucleus shape (Figure 3.5a,b,d). There was a significant increase in the space between fibers at each time point compared to fresh tendons (until ~300% at day 10) on the basis of the quantitative analyses. Moreover, there was a significant decrease in fiber density after 6 and 10 days of SD compared to fresh tendons (to ~85% and ~75%, respectively). Nucleus roundness of tendons after 4, 6, and 10 days of SD also increased significantly compared to that of fresh tendons.

The cell number of fresh tendons was found to be 251.9 ± 19.7 #cells/mm2 before normalization. As shown in Figure 3.5c, cell density first increased to 113% and 146% at days 2 and 4, respectively. The cell density then decreased to 98% and 65% at day 6 and 10 of SD, respectively. The cell density at day 10 was significantly different from those on day 0 and day 4.

Collagen tortuosity increased with culture time, as evaluated by the scoring technique. As shown in Figure 3.5e, this increase was significantly different at days 4 and 10 of SD compared to day 0.

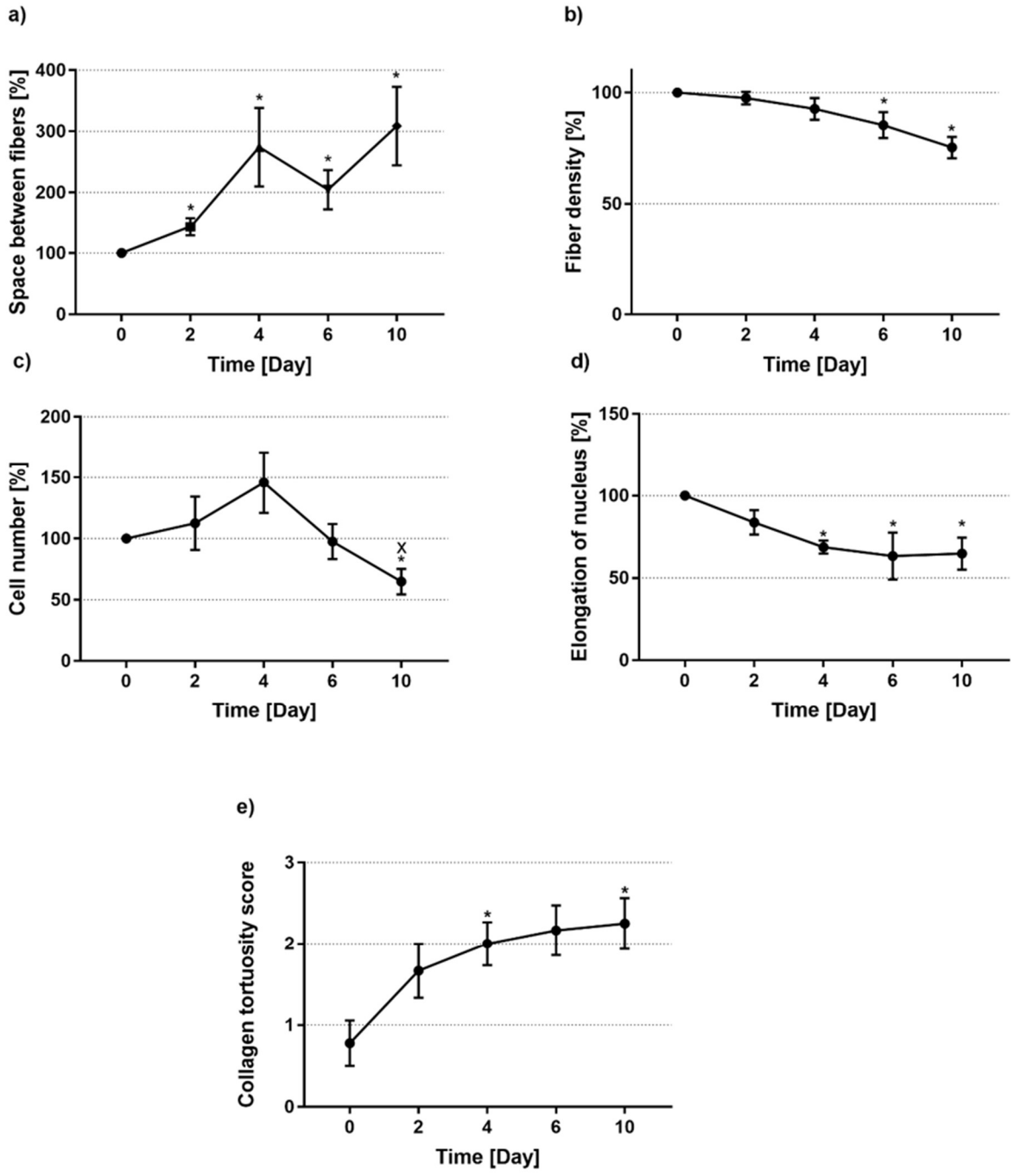

Figure 3.5 Quantification of a) space between fibers, b) fiber density, c) cell number, d) nucleus elongation, and e) semiquantification of collagen tortuosity. The data are presented as the means and SEM. * indicates a significant difference ($p<0.05$) from day 0 (fresh tendons); and x, a significant difference ($p<0.05$) from 4 days of stress deprivation.

Biochemical results

SD tendons demonstrated an upregulation of MMP13 with increasing SD time compared to fresh tendons (Figure 3.6a, and b). Epifluorescent staining data revealed that this upregulation was statistically significant at day 6 compared to the fresh tendons (Figure 3.6b). However, at day 10, there was a decrease in MMP13 level to ~130% compared to that at the previous SD time point (day 6).

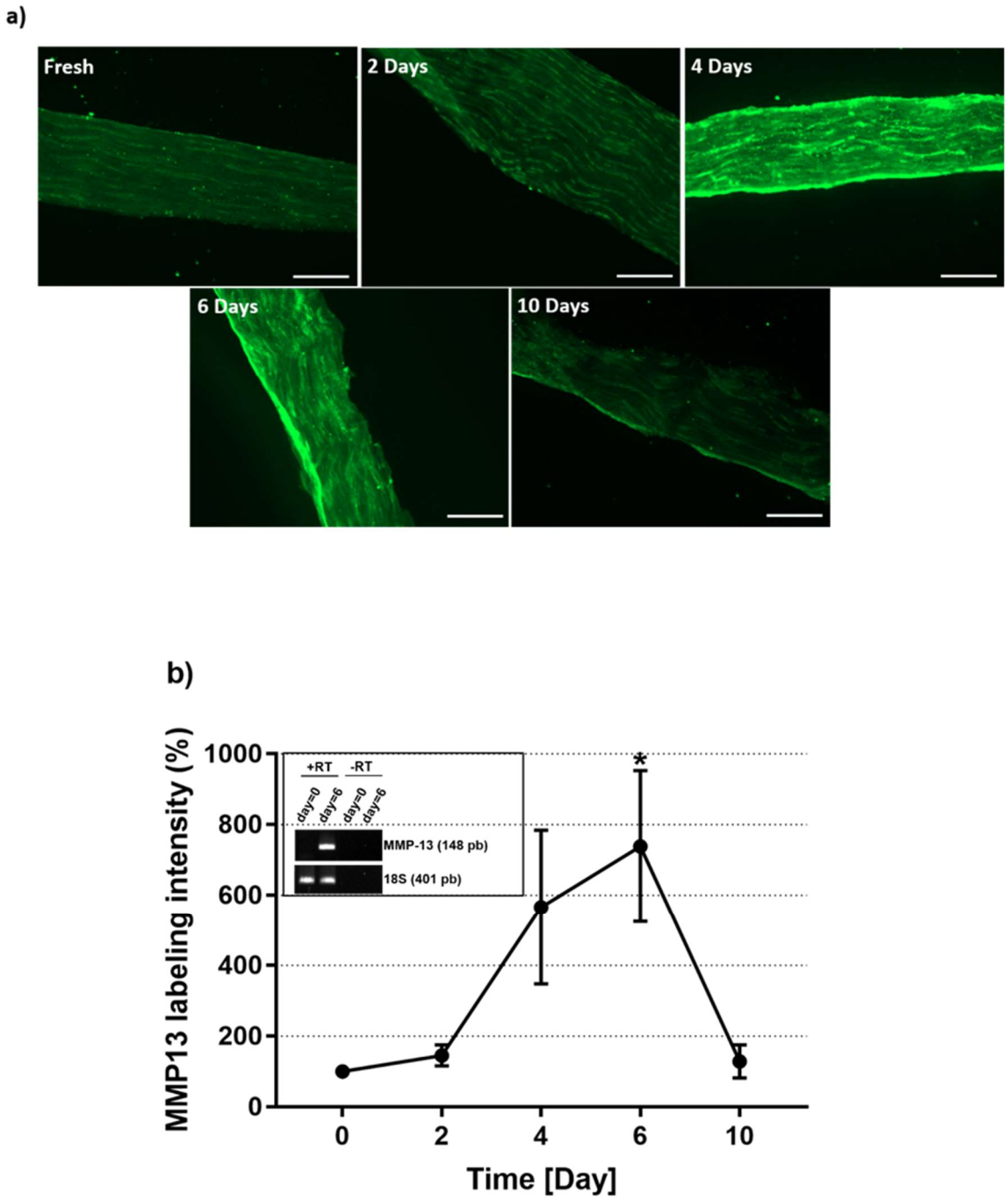

Figure 3.6 Biochemical analysis. a) Representative epifluorescence micrographs of tendons stained for MMP13 protein. Bar = 200μm. b) MMP13 labeling intensity evaluated for freshly isolated tendons as well as tendons that experienced 2, 4, 6, and 10 days of stress deprivation (means and SEM). Inset. Consolidation of increased MMP13 in one tendon sample by PCR analysis. * indicate significant difference ($p<0.05$) from day 0 (fresh tendons).

3.5. DISCUSSION

We developed an *ex vivo* tendinopathy model in RTTs using SD. After 4 days of SD under tissue culture conditions, we observed some moderate histopathologic, biochemical and viability changes but no changes in mechanical properties. We refer to this tendinopathy stage as moderate tendinopathy. This model will be useful for studying the progression of physiopathology in mechanobiological studies as well as for testing potential treatments to stop or reverse the progression of the pathology.

In both *ex vivo* and *in vivo* experiments, an increase in cell number has been reported in tendinopathic tendons[33,90,96,102,108]. This hypercellularity appears to be the initial response of tendons to activate their repair mechanism[106]. However, cellularity decreases with further progression of tendinopathy[90,102]. This results in an inverted-U curve relating cellularity with time. On this graph, we found that peak cellularity occurs at 4 days of SD, which we refer to as moderate tendinopathy. In contrast to these observations, *in vivo* SD of rabbit patellar tendons resulted in a progressive increase in cells over a 6-week period[108]. One reason explaining these observations could be differences in the underlying mechanisms of tendinopathy between *ex vivo* and *in vivo* models.

Our observations show that biomechanical properties are affected by SD only after approximately 5 days of culture time. Previous *ex vivo* studies have reported that short-term SD does not affect biomechanical properties, which is in accordance with our results. However, there is a lack of information in the literature regarding the time point at which this significant degradation in mechanical properties occurs. Our data suggest that mechanical deterioration happens between days 4 and 6 of SD. The observed delay in mechanical response to SD could be related to the absence of mechanical degradation, i.e., degradation through mechanical loading, in disuse models of tendinopathy development.

One of the pathologic characteristics that tendons experience during tendinopathy development is cell apoptosis[29,102,110]. In agreement with the literature, we observed a decrease in live cells and an increase in dead cells in both short-term and long-term tendinopathies. However, we did not specifically look at the type of cell death. In addition

to the results from short- and long-term SD, the current study also provides data from mid-term SD. In the current study, the results from day 4 of SD confirm that there is a continuous increase in cell death throughout the early, moderate, and advanced tendinopathy stages.

There is also evidence that tendon cells transform from an elongated shape to a round shape in tendinopathic tendons[106], in agreement with our observations. Based on previous *ex vivo* studies, cell roundness appears only after 24 hours of SD[6] and increases with culture time. Our data suggest that this increase in early and advanced tendinopathies is continuous through moderate tendinopathy as well.

Our data suggest that in addition to cellular shape, density and viability, gene response is also affected by SD. In agreement with our results, increases in MMP13 (mRNA and protein) have been consistently reported in tendinopathy[5,6,30,34,106]. In the current study, despite the initial increase, we observed a decrease in MMP13 levels after 10 days of SD. This decrease may be due to a decline in cell density and/or cell viability after 10 days of SD.

We observed a progressive decrease in fiber density of SD tendons from early (short-term) to advanced (long-term) tendinopathy. This decrease in fiber density of tendons has also been reported in previous *ex vivo* studies examining short- and long-term SD[5,30,102]. An increase in the space between fibers was also reported at both time points[30,102].

Usually, changes in fiber density and the space between fibers are accompanied by disorganized collagen bundles[106]. Our data suggest a continuous decrease in collagen alignment through early to moderate and advanced tendinopathy.

Currently, there is no single model available that represents all aspects of human tendinopathy[15,27]. Similar to other models, SD models have limitations. During SD, tendon properties are altered without exposing the tendon to mechanical loading. For this reason, SD models do not include simultaneous enzymatic and mechanical degradation. Accordingly, the model used in this study should be used with caution when investigating overuse injuries. SD models relate more closely to immobilization scenarios such as

casting or rest after surgery. Indeed, applying mechanical loads to SD tendons has been used to deepen our understanding of rehabilitation in these situations[102]. Finally, since SD models are easy to implement, they could be an excellent choice for studies in which multiple samples and conditions are needed. It should be noted that our SD system and obtained results were duplicable for 4-6-month-old rats. However, it is probable that changing the species, race, sex or age of the animals would alter some of the results, such as the time scale of the tendinopathy development. This could be the subject of future investigations.

In conclusion, we have created a model to develop moderate tendinopathy in RTTs using the SD method. To our knowledge, this is the first study to characterize the moderate stage of tendinopathy. Our data suggest that moderate tendinopathy occurs around day 4 of stress deprivation. Because advanced tendinopathy can be more difficult to cure and because early tendinopathy is often asymptomatic, meaning that patients most likely do not seek medical treatment, it is reasonable to test treatments for moderate tendinopathy first and, if successful, to proceed with treatments for more advanced tendinopathy. Our study characterized a moderate stage of tendinopathy in an *ex vivo* SD model based on the viability, histopathology, biochemical, and mechanical properties of tendons.

Chapter 4. Efficacy of combining PRP and MMP inhibitors in treating moderately damaged tendons *ex vivo*

4.1. ABSTRACT

Platelet-rich plasma (PRP) and broad-spectrum matrix metalloproteinase inhibitors (MMPIs) have been used as therapeutic options for tendinopathy. However, mixed results have been reported regarding their efficacy. We posited that the combination of these two treatment strategies would be more beneficial for healing tendons than each treatment alone. Rat tail tendons were harvested and cultured without mechanical stress for 0, 4, or 10 days. Single and combination treatment with PRP and MMPIs was administered to 4-day stress-deprived (SD) tendons. This treatment was applied to the tendons over 6 days. At the end of their culture time, the tendons were subjected to traction testing and histopathological, biochemical, and viability assays. The results showed better mechanical and histological results for PRP+narrow-spectrum MMPIs (NI) than for PRP, NI, or broad-spectrum MMPIs (BI) alone. The stress at failure of the PRP+NI-treated tendons was better than that of the 10-day SD tendons. Compared with the 10-day SD tendons, the PRP+NI-treated tendons also had improved fiber density, nucleus shape, and space between fibers. This study shows that the combination of PRP with NI is a potentially effective treatment approach for tendinopathy.

Keywords: platelet rich plasma; matrix metalloproteinase inhibitor; mechanical properties; biochemical properties; histology

4.2. INTRODUCTION

Tendinopathy, a general condition of tendon disorder, is a common problem, particularly for athletes and workers. To date, the optimal strategy for treating tendinopathy is still not defined. Platelet-rich plasma (PRP) and matrix metalloproteinase inhibitors (MMPIs) are regarded as two promising treatments. Modeling the tendon mechanobiological response (TMR) as

$$\text{TMR} = \text{Repair} - (\text{Mechanical degradation} + \text{Enzymatic degradation})^{22} \quad (4.1)$$

highlights that the combination of these treatments might be beneficial by enhancing tendon repair while inhibiting the proteolytic degradation of connective tissues.

Administration of autologous PRP at lesion sites has been shown to promote tendon regeneration. This positive effect of PRP is believed to result, at least in part, from growth factors released from activated platelets[16,93,112].

Indeed, there is a large body of preclinical and clinical evidence supporting the beneficial effects of PRP therapy on tendinopathy[16,20,35,43,58,59,67,93,95,97,103,104]. The beneficial effects of PRP therapy may include tenocyte proliferation[3,11,36,58,68] and improved morphology[93], increased collagen production and organization[16,43,56–58,89], increased mechanical strength[16,43,58,93,97,104] and a better repair rate[56,57,59]. However, some studies did not observe any positive effect of PRP on damaged tendons[82,87,92]. It has been suggested that the presence of catabolic proteases such as matrix metalloproteinases (MMPs) in PRP could inhibit tissue healing[82].

MMPs are a family of enzymes responsible for extracellular matrix (ECM) degradation[69] and could play an important role in tendinopathy development[24,78,86]. An increase in the MMP level in tendinopathic tissue has been reported in many studies[23,61,73]. Therefore, inhibiting MMPs has been considered a potential healing approach to stop the pathological process of tendinopathy[23,24,60]. Greater collagen organization[13,14,25,44,70], as well as a reduction in collagen degradation[14,70], limited MMP activity[13,25,44,70], and increased mechanical strength[13,25,44,70], have been reported as effects of the use of broad-spectrum

matrix MMPIs (BI) (e.g., doxycycline and alpha-2-macroglobulin) in surgically induced tendinopathy models. MMPIs have also been used in clinical trials to improve tendon healing. The most common conditions that have been treated by injection of the MMPI aprotinin in clinics are Achilles, patellar, and hamstring tendinopathies[73]. These studies have yielded mixed results regarding the effectiveness of this approach[9,17,19]. Some studies have shown that broad-spectrum MMP inhibition impairs tendon healing, since some MMPs play a fundamental role in remodeling and healing[46,54,75,79]. Therefore, more selective inhibition might lead to positive effects. In fact, it would be beneficial to target specific MMPs that are detrimental to tendon healing without affecting MMPs that either are beneficial for tendon remodeling or cause side effects when inhibited[62]. This targeting could be achieved by applying narrow-spectrum MMPIs (NI) instead of BI. Based on the literature highlighting the detrimental roles of the upregulated MMP13 in the etiopathogenesis of tendinopathy[5,6,34,75], the selection of NI was oriented towards a commercially available, cell-permeable, small molecule inhibitor specific to MMP13, referenced under CAS 204140-01-2 (IC_{50}=900 pM).

Based on the above considerations, the present study was designed to investigate the efficacy of PRP and MMPIs (BI and NI), alone or combined, to treat moderately damaged tendons, i.e., 4-day stress-deprived (SD) tendons, *ex vivo*. Our objective was to investigate whether combination of PRP and MMPIs provides better results than PRP or MMPIs alone.

4.3. MATERIALS AND METHODS

Animals. Experiments were carried out on male Sprague-Dawley rats (4- to 6-month-old) (Charles River; St. Constant, Québec, Canada). Animal housing and the experimental protocol (protocol #EL2014-03) were carried out in accordance with regulations set by the Canadian Council of Animal Care Committee and were approved by the Animal Care Committee of the University of Sherbrooke (CIPA/CFPA-FMSS).

Rat tail tendon (RTT) extraction. Tendon isolation and preparation were conducted as described in a previous study[18]. The distribution of rats and tendons for mechanical tests and viability, histological and biochemical analyses is shown in Figure 4.1. All

manipulations were performed in a cold Dulbecco's phosphate-buffered saline (D-PBS) solution (311-410-CL; Wisent Inc., St-Bruno, Canada) containing 1 g/L glucose (609-037-EL; Wisent Inc.) and 1% antibiotic-antimycotic (15240-062; Invitrogen, Burlington, Canada). Following isolation, tendon cross-sectional areas were evaluated using a custom optical micrometer and a stereomicroscope[76]. Briefly, the tendons were maintained in the saline solution to avoid dehydration and were rotated along their longitudinal axis. Images of specimen projections were captured at 10° angular increments, and the tendon edges were localized within a local reference frame using a contrast-based image analysis algorithm. The cross-sectional areas were estimated using a profile reconstruction algorithm. The tendons were then washed 5 times under sterile conditions. Afterwards, the tendons were transferred to tissue culture flasks containing DMEM solution (12800-017; Invitrogen, Burlington, Canada) supplemented with 3.7 g/L sodium bicarbonate (600-105-CG; Wisent Inc.), 10% FBS (090150; Wisent Inc.), and 1% antibiotic-antimycotic.

RTT incubation. Tendons were divided into three main groups: 1) Fresh, 2) SD, and 3) treated (Figure 4.1). The fresh tendons were the newly extracted tendons.

The SD tendons were tendons cultured without mechanical loading to create moderate or advanced tendinopathy. These tendons were divided into two groups and incubated in tissue culture conditions (37 °C, 95% humidity, 5% CO2, sterile environment) for 4 or 10 days. The medium was changed every 2 days.

Treated tendons were first incubated in tissue culture conditions (37 °C, 95% humidity, 5% CO2, sterile environment). For 4 days, these tendons were cultured without mechanical loading to create moderate tendinopathy, and the medium was changed every 2 days. On day 4, 1.5 MPa static loading (SL) was applied to the moderately damaged tendons by attaching a stainless steel weight to an approximately 11 cm long RTT in order to stop the progression of the tendinopathy at the time of treatment application. This loading amplitude was chosen based on the reported anticatabolic effect of low SL on tenocytes in the literature[8,107] and our results from preliminary tests (data not shown). For 6 more days, the loaded tendons were suspended in polyethylene tubes containing

the mentioned culture medium with or without biochemical treatments: PRP, NI, BI, PRP+NI, and PRP+BI (Figure 4.1) using the following concentrations: 10 nM NI (CAS 204140-01-2, Millipore Sigma, Ontario, Canada), 25 μM BI (CC1010, Millipore Sigma, Ontario, Canada), and 2.5% (vol/vol) PRP. The medium was changed once, 3 days after the treatment application (corresponding to day 7 of the experiment). There were two tendons in each subgroup, one for mechanical characterization and one for viability, histological and biochemical studies.

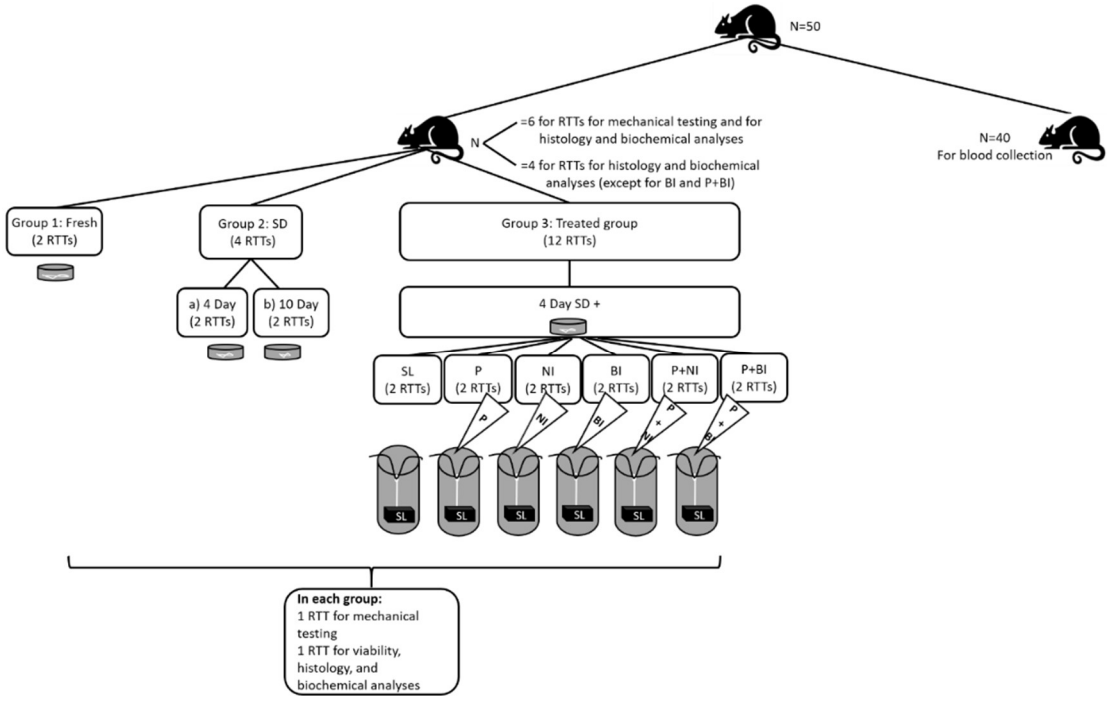

Figure 4.1 Number and distributions of tendons from each rat. RTT stands for rat tail tendon. SD stands for stress-deprived. N stands for the number of rats for either RTT extraction or blood collection. 4 Day and 10 Day indicate 4 and 10 days of stress deprivation, respectively. SL, P, NI, BI, P+NI, and P+BI stand for static loading, platelet-rich plasma (PRP), narrow-spectrum matrix metalloproteinase (MMP) inhibitor, broad-spectrum MMP inhibitor, PRP+narrow-spectrum MMP inhibitor, and PRP+broad-spectrum MMP inhibitor, respectively. There were two tendons in each group, one for mechanical analysis and the other for viability, histological, and biochemical analyses.

Blood collection and PRP preparation. For each experiment, whole blood was collected from 4 male 4-6-month-old Sprague-Dawley rats, 2 rats at day 4 and 2 rats at day 7 of the experiment. The rats were anesthetized with ketamine/xylazine (1 µl/g, intramuscular), and 12-14 ml of whole blood was collected by cannulation of the carotid artery with a polyethylene catheter (PE-10) containing heparinized saline solution. Blood was collected in Eppendorf tubes containing 3.5% sodium citrate via the carotid artery. PRP was obtained from blood samples by low-speed centrifugation (100g, 20 min, room temperature). The platelets inside the PRP were activated by adding adenosine diphosphate (ADP) (A2754, Sigma-Aldrich, Milwaukee, USA) to the PRP immediately before adding PRP to the culture medium. The activated PRP was immediately applied to the culture medium at a 2.5% concentration.

Platelet counts were conducted using a hemocytometer shortly after PRP preparation. The required concentration was approximately 1×10^6 platelets/µl PRP. PRP samples with a lower platelet concentration were discarded from the experiment.

RTT mechanical characterization. Mechanical tests were conducted on live tissues at the end of their culture time (i.e., day 0 for group 1, day 4 or 10 for group 2 and day 10 for group 3). At the time of mechanical testing, each end of the tendon was aspirated into one 2-3 cm long silicon tube using a microvolume pipette. To prevent tendon sliding, the tubes were compressed using clamps and were fixed inside the testing compartment. For mechanical characterization, the tendons were transferred to a custom-manufactured bioreactor[77]. The specimens were subjected to the following testing protocol. The initial zero-strain reference value was defined as the strain when a tension load of 3 g was reached at equilibrium. Preconditioning was performed with a series of 120 sinusoidal waves at two different amplitudes (60 cycles at 1% strain; 60 cycles at 2% strain) at 1 Hz. The final zero-strain reference value was defined again as the strain when a tension load of 3 g was reached at equilibrium. Afterwards, the tendons were subjected to a traction test (at a strain rate of 0.5%/s). The maximum engineering stress before failure was reported as the measure of the tendon strength. The data normalized to the day 0 values are reported.

Viability test. Viability tests were conducted on tissue at the end of their culture time (i.e., day 0 for group 1, day 4 for group 2 and day 10 for group 3). The viability of cells from the fresh and cultured tendons was assessed using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (L3224; Invitrogen, Carlsbad, CA). Tendons were washed in D-PBS to remove any serum and then incubated with ethidium homodimer-1 (1 μM for 45 min) followed by treatment with calcein AM (1 μM for 40 min) in D-PBS at room temperature. The tendons were washed briefly in D-PBS prior to mounting on glass slides. Green-fluorescing live cells and red-fluorescing dead cells were visualized within 45 min with an EVOS FL Auto microscope (Thermo Fisher Scientific, Massachusetts, USA) and recorded with a digital camera.

Immunofluorescence staining of MMP13 protein. Immunohistochemical studies of MMP13 were conducted on tissue samples previously fixed in 10% neutral buffered formalin (032-060, Fisher Scientific Inc., Kalamazoo, MI) and embedded in optimal cutting temperature (OCT) embedding medium. The embedded tissues were longitudinally sectioned to 20 μm thickness using a cryostat (Thermo Scientific, Cheshire, UK). The sections were incubated in D-PBS containing 0.1 M glycine (56-40-6, Roche Diagnostic GmbH, Mannheim, Germany) for 10 min to wash away the mounting medium used for tissue sectioning. The sections were incubated in D-PBS containing 0.1% Triton (T8787, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 2% bovine serum albumin (BSA) (A7030, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at room temperature (RT) for 30 min inside a humid chamber. The sections were then washed once with D-PBS containing 0.1 M glycine and five times with D-PBS. Subsequently, the sections were incubated in rabbit polyclonal anti-MMP13 antibody (ab39012, Abcam, Cambridge, UK, 20 μg/μl, all antibodies were diluted in D-PBS containing 0.05% Triton and 1% BSA) and DAPI (62248, Sigma-Aldrich, Oakville, Canada, 1 μg/ml) at RT for 1 hour. The sections were washed five times with D-PBS and were incubated in secondary antibody (A11034, Alexa Fluor 488 goat anti-rabbit IgG, Life Technologies, Carlsbad, California, USA; 20 μg/μl) at room temperature for 1 hour. After further washing in D-PBS, sections were incubated in D-PBS containing 0.1% Triton and 2% BSA at room temperature for 10 min. The sections were then washed in D-PBS, mounted on microscopy slides with mounting

medium (F4680, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and sealed with nail polish. The negative controls were sections incubated with rabbit isotype IgG antibody (X0903, Agilent Dako, Santa Clara, USA; 20 µg/µl, diluted in D-PBS containing 0.05% Triton and 1% BSA) without being exposed to primary antibody. Green (MMP13 proteins) and blue (nucleus) fluorescence images were captured with the EVOS FL Auto microscope (Thermo Fisher Scientific, Massachusetts, USA).

The 20× images of MMP13 protein were analyzed with Image-Pro Plus software (version 6.0.0) to quantify the MMP13 intensity. From each image, three regions of interests (ROIs) were selected, taking care to exclude the tendon periphery (which usually has saturated signals). The MMP13 intensity was calculated as the summation of the signal densities signals in each pixel of each ROI. The results from the three ROIs were averaged.

Histological characterization. Histological studies were conducted on biopsies that were taken from each tissue, fixed in 10% neutral buffered formalin and embedded in either paraffin for hematoxylin and eosin (H&E) staining or in OCT medium.

Paraffin-embedded biopsies were longitudinally cut into 6 µm-thick sections and were processed in H&E stain for light microscopy observation. Images of each sample were captured using a NanoZoomer whole slide imaging system (Hamamatsu Photonics K.K., Hamamatsu, Japan) in visible mode. The 20× images were analyzed using the following five parameters: fiber density, areas of the spaces between fibers, fiber tortuosity, tenocyte density and nucleus shape.

The two first parameters of the ECM were evaluated using Image-Pro Plus software as described previously[38]. For each micrograph, three ROIs were captured taking care to exclude regions damaged by the cutting blade. The ROIs were then analyzed with Image-Pro Plus software. Data normalized to day 0 are presented.

Fiber density. The background density was found by contrast and separated into two categories: background (red) and fibers (black). The fiber density was calculated as:

$$Fiber\ density\ (\%) = (1 - \frac{Number\ of\ pixels\ of\ background}{Number\ of\ pixels\ of\ ROI}) * 100\% \qquad (4.2)$$

The results from the three ROIs were averaged.

Areas of the spaces between fibers. For each ROI, the areas of the spaces between fibers were also evaluated in terms of pixel number and then averaged. Data normalized to day 0 are reported.

Tenocyte density. Tenocyte density was evaluated as:

$$Tenocyte\ density = \frac{Number\ of\ tenocytes}{Tendon\ longitudinal\ area} \quad (4.3)$$

Using a grid and the known scale bar, the number of cells were counted, and the tendon longitudinal area was calculated.

Nucleus shape. Longitudinal cryo-sections of formalin-fixed biopsies 20 μm in thickness were stained with DAPI (DAPI, Sigma-Aldrich, 62248, 1 μg/ml) (as described in the biochemical characterization section). Images of blue nuclei were captured using the NanoZoomer whole slide imaging system. The 40× images were analyzed using Image-Pro Plus software. The nucleus shape was recognized by contrast, and its roundness was evaluated as:

$$Nucleus\ roundness = \frac{Perimeter\ of\ nucleus^2}{4*\pi*Area\ of\ nucleus} \quad (4.4)$$

A value of 1 indicates a perfect circle, whereas higher values indicate more elongated shapes. Data normalized to day 0 are reported.

References for comparison

We chose three references to evaluate the results of the applied treatments: 1) Fresh tendons as they represent the optimal goal for tissue repair; 2) 4-day SD tendons (tendinopathic models)[39] as they represent the tissue state we treated and wanted to improve; and 3) 10-day SD tendons as they represent the progression of the untreated tendinopathic model that should at least be improved by the treatments. In other words, the optimal treatment would improve the tendinopathic tendons to the quality level of fresh tendons. At the other end of the spectrum, a less optimal treatment should not

degrade the tendinopathic tendons to a state worse than 10-day SD tendons. Other treatments, such as stabilizing or slowing down the degradation, would be classified in between these effects.

Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM). Biomechanical, histological, and biochemical results were compared using nonparametric Wilcoxon matched-pairs signed rank tests (GraphPad prism, 7.03) or one-sample Wilcoxon tests (IBM SPSS) comparing the groups to day 0. Significance was set at p values <0.05.

4.4. RESULTS

Cross-sectional area

The cross-sectional areas of the tendons were calculated to be 0.037 ± 0.002 mm^2 (mean ± SEM).

Platelet concentration

The platelet concentration within PRP was evaluated to be $1.36 \pm 0.09 \times 10^6$ platelets/µl.

Cell viability

Figure 4.2 shows representative fluorescence micrographs of the tendons. Fresh samples (group 1) showed a high number of green cells and a few red cells, indicating a high level of viability. As the time of the SD increased (group 2), i.e., 4-day SD and then 10-day SD, the number of red cells in the samples gradually increased.

For treated tendons (group 3), qualitative observations suggest that the cell viability was comparable to that of the 4-day SD tendons. No evident differences were observed between the treated tendons.

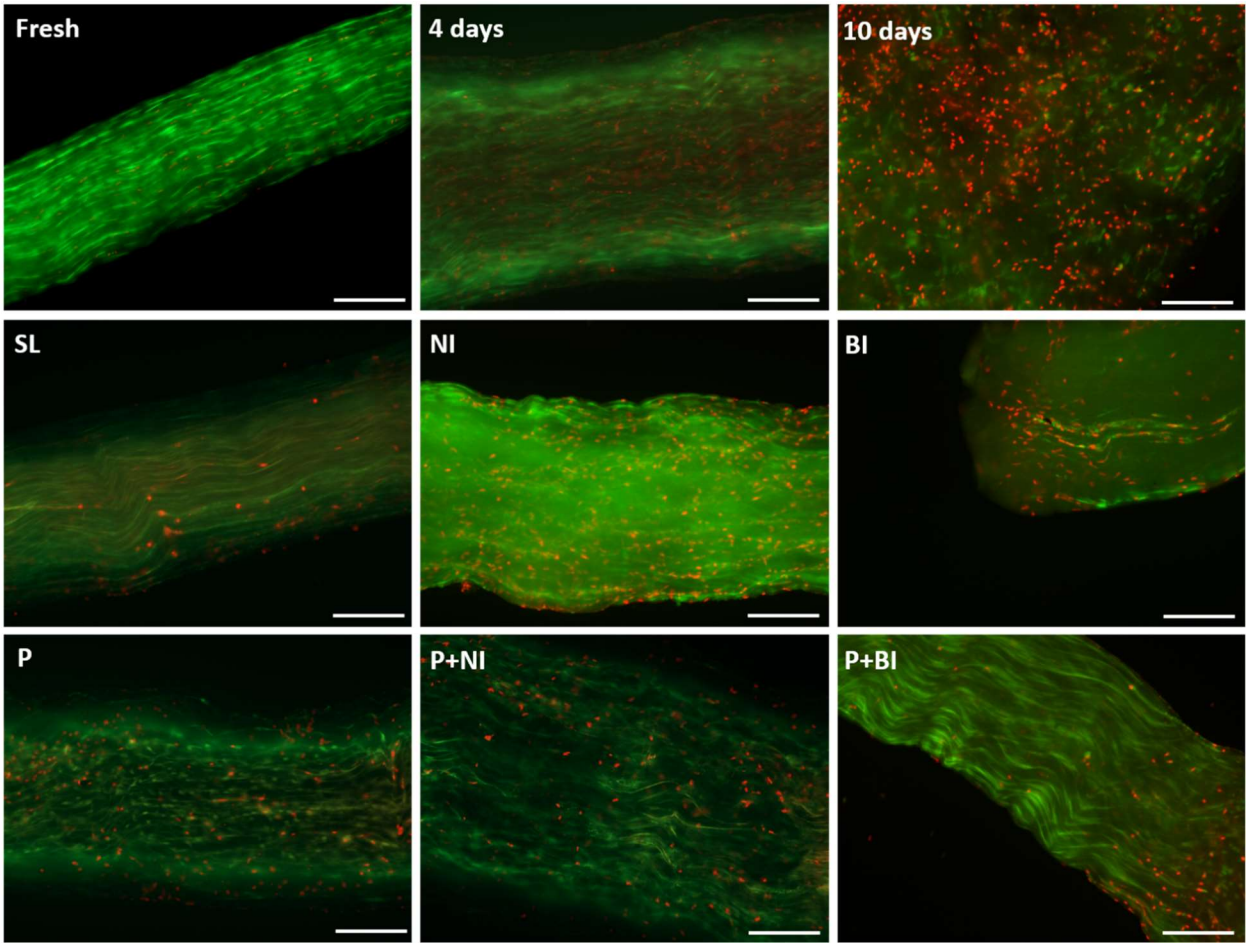

Figure 4.2 Representative fluorescence micrographs of tendons in the various treatment groups. Abbreviations as in figure 1. Green-fluorescing cells are alive, and red-fluorescing cells are dead. Bar=200 μm.

Mechanical result

The maximum stress at failure of fresh tendons was evaluated to be 8.54 ± 0.83 MPa (mean ± SEM). Consistent with our previous result (unpublished data), on day 10, the stress at failure of the SD RTTs (group 2) decreased significantly to ~ 65% of the value for fresh tendons (Figure 4.3). For the treated tendons (group 3), the stress at failure of the PRP+NI-treated tendons was ~ 95% of the value for fresh tendons. This treatment was the only treatment that improved the mechanical strength of our tendinopathic model to a value better than that of 10-day SD tendons (p=0.1). The other treatments resulted in a reduction in the stress at failure to ~ 50% or less relative to that of the fresh tendon.

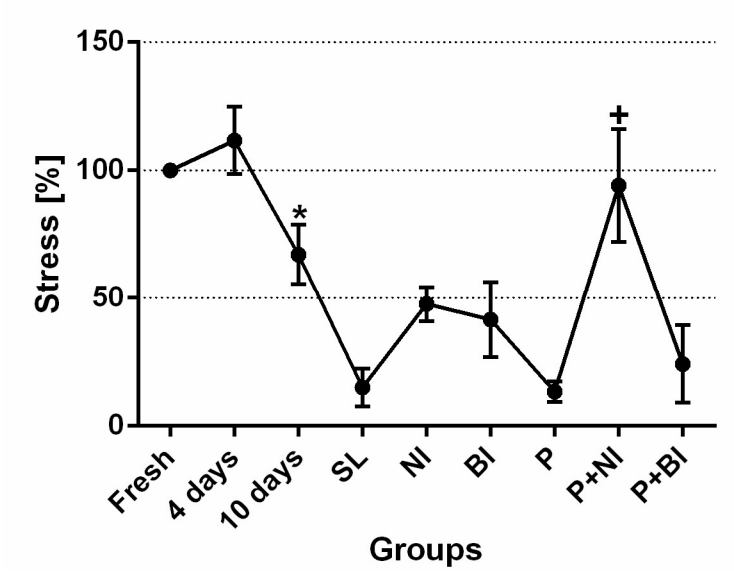

Figure 4.3 Changes in maximum stress at failure (mean and SEM). Abbreviations as in figure 1. * indicates p<0.05 compared to fresh tendons, and + indicates p=0.1 compared to 10-day SD.

Histology

The fiber density and the space area between fibers of the fresh tendons were evaluated to be 94 ± 1%, and 54.9 ± 8.6 pixels (mean ± SEM), respectively. Under a light microscope, freshly harvested samples showed dense and well-aligned collagen fibers (Figure 13). Compared to fresh tendons, the 4-day SD tendons showed a significantly lower fiber density and a larger space area between fibers (Figure 13 and Figure 14a and b). A further decrease in fiber density and an increase in the space between fibers was observed in the 10-day SD tendons (group 2) compared to the fresh and 4-day SD tendons. These changes

relative to the fresh tendons were significant. In the treated tendons (group 3), the SL-, PRP-, and NI-treated tendons showed significantly lower fiber density than fresh tendons. Compared to the 4-day SD tendons, none of the treated tendons had significantly different fiber density or space between fibers. However, compared to the 10-day SD group, the PRP- and PRP+NI-treated groups had a significant improvement in fiber density. Moreover, the space between fibers was significantly lower in the PRP+NI-treated group than in the 10-day SD group (Figure 4.4, and 4.5a and b).

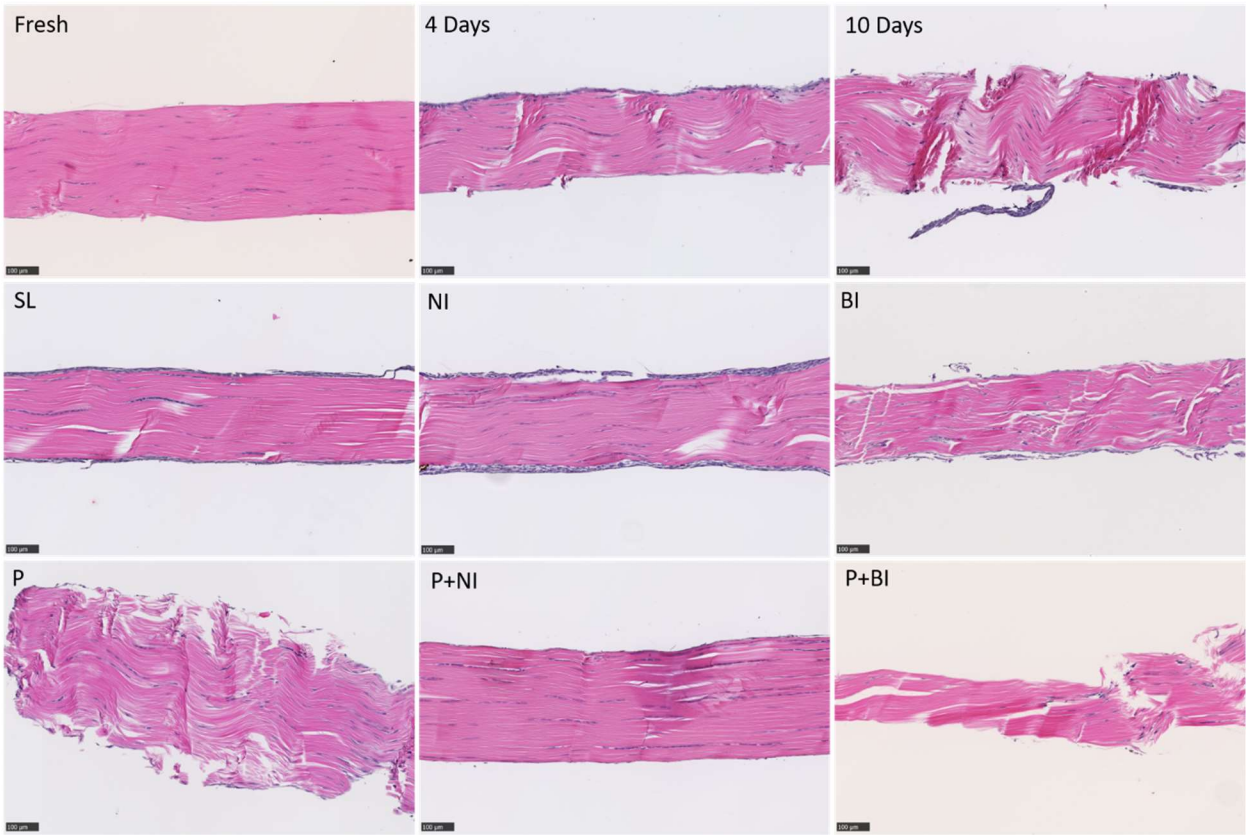

Figure 4.4 Typical micrographs of longitudinal sections of tendons stained with hematoxylin and eosin. Abbreviations as in figure 1. Bar=100 μm

The cell number of fresh tendons was evaluated to be 220 ± 21 cells/mm^2. The cell density was significantly higher in 4-day SD tendons than in fresh tendons (Figure 4.5c). However, in 10-day SD tendons, the cell density returned to ~ 104% of the cell density observed in fresh tendons (Figure 4.5c). The cell density of 10-day SD tendons did not differ significantly from that of 4-day SD or fresh tendons. In the treated tendons (group 3), only the PRP+BI treatment resulted in a significant increase in cell density relative to that in fresh tendons. The other treatments did not significantly change the cell density from that in fresh or 4-day SD tendons. Compared to 10-day SD tendons, PRP- and PRP+BI-treated tendons had significantly higher cell density (Figure 4.5c).

The nuclear roundness of fresh tendons was evaluated to be 2.34 ± 0.16 (mean ± SEM). Compared to the cells in fresh tendons, the cells in both 4- and 10-day SD tendons became significantly less elongated and became round (Figure 4.5d). In group 3, the SL-, NI-, and PRP+BI-treated tendons also showed significantly rounder cells than the fresh tendons. None of the treatments caused the shape of the tenocytes to differ significantly from the shape of the cells in 4-day SD tendons. However, compared to the 10-day SD tendons, the SL-, PRP-, and PRP+NI-treated tendons had a significantly more elongated cell shape (Figure 4.5d).

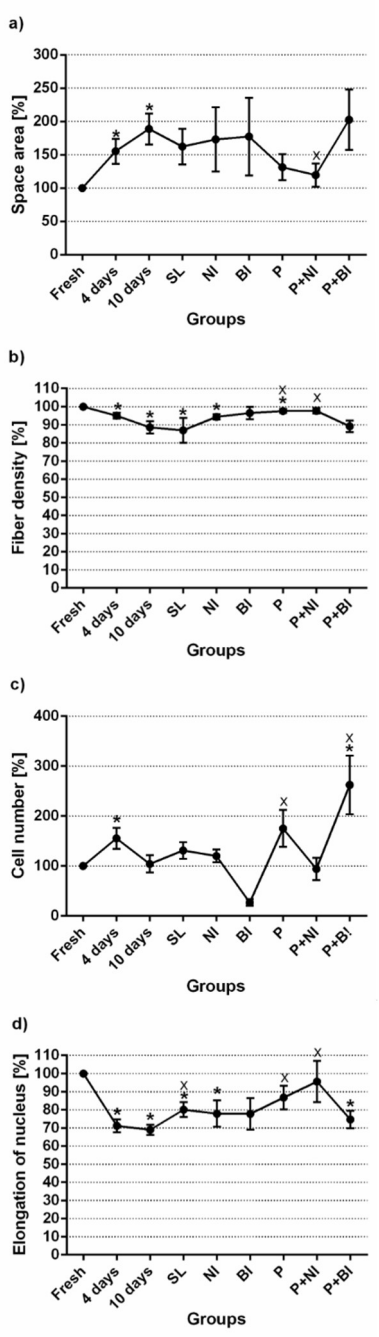

Figure 4.5 Quantification of the a) space between fibers, b) fiber density, c) cell number, and d) nucleus elongation. Abbreviations as in figure 1. The data are presented as the mean and SEM. * indicates a significant difference ($p<0.05$) compared with fresh tendons; x, a significant difference ($p<0.05$) compared with 10-day SD tendons.

Biochemical results

The 4-day SD tendons had an approximately similar MMP13 level as the fresh tendons (Figure 4.6). However, in the 10-day SD tendons, the MMP13 level significantly decreased to ~ 48% of the MMP13 level of fresh tendons. None of the treatments resulted in significant changes in the MMP13 level comparing to those of the fresh, 4-, or 10-day SD tendons. While the MMP13 level of all treated groups remained approximately 250% or less, the MMP13 level of the PRP-treated group was found to be ~ 800%.

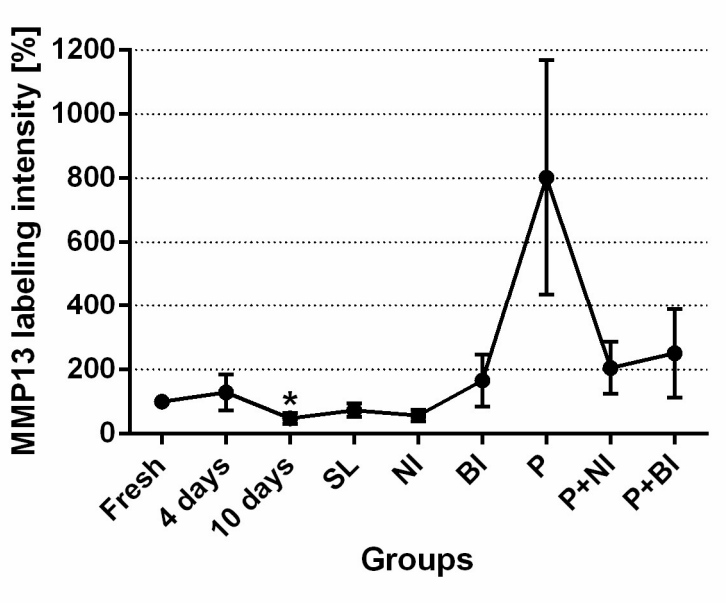

Figure 4.6 Biochemical analysis. MMP13 labeling intensity evaluated for freshly isolated tendons, SD tendons and treated tendons (mean and SEM). Abbreviations as in figure 10. * indicates $p<0.05$ compared to fresh tendons.

4.5. DISCUSSION

We investigated the efficacy of PRP and MMPIs (BI and NI) alone and in combination using an *ex vivo* model of moderate tendinopathy. We showed that PRP combined with a narrow-spectrum MMP inhibitor is more efficient in treating moderate tendinopathy *ex vivo* than PRP or MMPIs alone. Moreover, our data suggest a trend for better histological and mechanical results with NI than with BI, when NI or BI are combined with PRP.

The improved healing by the combination of PRP and MMPIs is probably associated with a simultaneous improvement in regeneration (i.e., molecular events resulting in matrix synthesis and tissue repair) and inhibition of selected degeneration processes (i.e., events resulting in matrix degradation). Comparing the results from PRP+NI and PRP+BI reveals that cell number and nucleus shape of PRP+NI-treated tendons are comparable to those of healthy tendons but not PRP+BI-treated ones. This observation together with the mechanical results could reflect a synergistic effect between PRP and NI. However, further studies are needed to demonstrate and understand the effect.

There has been growing evidence indicating that using NI rather than BI would lead to greater efficacy and less adverse effects in different diseases, such as cancers and arthritis[12,62,109]. Although the exact mechanisms of these BI-induced side effects are poorly understood, it has been suggested that the lack of selectivity of these inhibitors could be a reason[109].

It has already been shown that PRP could have beneficial effects in treating tendinopathy, but the results varied. PRP has some catabolic and inflammatory effects on tendon cells, such as catabolic protease (e.g., MMP) production, which might be detrimental to tissue healing[82,113].

For PRP-treated tendons compared to 10-day SD tendons, our data revealed improved fiber density and tenocyte morphology. Despite these improvements in histology, no improvement in the mechanical properties of PRP-treated tendons was observed. In the literature, there are some discrepancies regarding mechanical results. Virchenko *et al*.

reported the positive effect of PRP on the mechanical properties of a rat Achilles tendon subjected to surgically induced tendinopathy after only 3 and 5 days of PRP treatment *in vivo*[97]. However, in most *in vivo* studies, PRP-treated tendinopathic tendons, which had either surgically induced[16,43,58,93] or collagenase-induced[20] tendinopathy, exhibited improved mechanical properties after 2 weeks or more but not at earlier time points[43,104]. Therefore, it is possible that 6 days was not sufficient for PRP to improve the mechanical properties and that mechanical improvement could have happened at later times.

Inhibiting MMPs (broadly and selectively) has also been considered as a therapeutic option for tendinopathy[23,24,60]. However, in this study, MMPIs (either NI or BI) alone did not improve the mechanical or histological properties of tendons. It should be noted that most of the studies that reported beneficial effects of MMPIs on tendon healing were conducted *in vivo* with tendinopathy created through surgical intervention[13,14,25,44,70]. This condition might explain the difference between our results and those studies. Moreover, in most studies, the tendons were analyzed at day 7 or a few weeks after drug application. Therefore, it is possible that the 6-day culture time in this study was not sufficient for the MMPIs to show efficacious results.

The mechanical results show that compared to 4-day SD tendons, low SL tendons had a lower maximum stress ($p=0.1$). This finding contradicts our presumption and previous reports in the literature, i.e., the application of low SL maintains tendon properties[1,107]. However, it should be noted that unlike studies in the literature, which examined the effect of low SL on fresh samples, we have applied SL to damaged tendons. It has been theorized[4] that partial collagen tears in damaged tendons prevent the tendons from properly transferring an external load to cells. The resulting understimulated cells produce more degeneration than repair. One might think that increasing the amount of SL would increase the load amount perceived by cells and could stop enzymatic degradation. However, in our preliminary tests, higher loads (2.5 MPa) led to tendon breaking in less than 48 hours. One possible solution could be applying higher SL with some rest periods. This process would probably trigger the cell repair response (SL) while minimizing mechanical degradation (rest periods).

In this study, we showed that PRP combined with NI is a potentially effective treatment for healing tendinopathy and leads to better mechanical and histological properties than the traditional use of PRP or MMPIs alone. These results suggest that PRP and NI combination therapy may be clinically useful. Further studies could investigate the effect of exposing tendons with chronic tendinopathy to a combination of PRP and NI for longer times, as well as applying an SL higher than 1.5 MPa with rest periods between loading episodes. Obviously, *in vivo* application of PRP and NI combination therapy is necessary to support the findings of this *ex vivo* research study.

Chapter 5. Conclusion

5.1. Summary

Tendon disorders are common health problems. They can affect several aspects of people's life from daily to work-related activities. Although, many strategies have been suggested to treat tendinopathy, none of them was completely satisfactory. Developing animal tendinopathy models (*in vivo* and *ex vivo*) not only would help to better understanding the pathology behind tendon damage, but also to find an optimal treatment for the disease.

We decided to characterize an *ex vivo* model in this study, considering our lab expertise and equipment. Moreover, in *ex vivo* models, unlike *in vivo* models, there are less difficulties regarding developing and reproducing the tendinopathy model. This is because, in *ex vivo* models, there is no need to interact with animal recurrently to make them exercise, for instance. Therefore, these models are cheaper and less time consuming. However, *in vivo* models could be the next step following *ex vivo* tendinopathy induction, since they are more complete and closer to the human context.

Among all available methods to develop tendinopathy *ex vivo*, we chose stress deprivation. Although SD models have already been used to create tendinopathy, there was a lack of knowledge regarding moderate stage of damage. Moderate stage of tendinopathy is crucial specially when it comes to investigating the efficacy of treatments. On the one hand, early stage of human tendinopathy is often asymptomatic[48,65]. On the other hand, advanced tendinopathy could be difficult to treat with non-surgical treatments. This is because of some irreversible pathological features such as cell apoptosis. Therefore, moderate tendinopathy could be optimal level of damage to investigate the efficacy of treatments.

To treat tendinopathy, a combination of PRP and MMPI was proposed in this study. These methods were chosen based on their promising results in the literature. Moreover, modeling the tendon mechanobiological response (TMR) as

$$TMR = Repair - (Mechanical\ degradation + Enzymatic\ degradation)^{22} \quad (5.1)$$

indicates that promoting repair via PRP, and inhibiting MMP-mediating degeneration via MMPI at the same time seem a complementary approach toward regaining tendon health. Our results indicated 4 days of SD is needed to characterize a moderate tendinopathy model, and combination of NI and PRP are the best when comparing to NI, BI, PRP, and combination of BI and PRP.

5.2. Original contribution

In the first part of this study (article 1), we characterized a SD model *ex vivo*. We observed some moderate pathologic changes in RTTs' histological, biochemical, and mechanical properties after 4 days of SD. These changes include increases in the space between fibers, cell density, and collagen tortuosity and a decrease in collagen density and elongation of cell nuclei. No alterations in the stress at failure of tendons were observed in the moderate stage of damage. It should be noted that the changes in these properties grew progressively from day 4 until day 10, except for cell density, and stress at failure. We observed a drop in cell density and stress at failure between days 4 and 6 of SD. This model will be useful for studying the progression of physiopathology in mechanobiological studies as well as for investigating the efficacy of treatments to stop or reverse the tendinopathy progression.

To our knowledge, this is the first study to characterize moderate stage of tendon damage. Moreover, for the first time, a combination of MMPI and PRP was a suggested as a treatment strategy.

In the second part of this study (article 2), we applied MMPI (NI or BI) and PRP, in combination or alone, on created moderate tendinopathy model *ex vivo*. Moreover, to stop the degradative effects of SD, we applied a low static load on all treated tendons. After 6 days of RTTs culture under treatments conditions, combination of PRP and NI resulted in superior histological and mechanical properties comparing to other treatments, and to non-treated condition.

We perceived a potential synergistic effect of combining PRP and MMPIs. We also observed an improvement in stress at failure, fiber density, nucleus shape and space between fibers in treated tendons comparing to untreated ones.

Finally, during the progression of this project, we developed a new method to fix the tendons inside bioreactor chambers (not presented in this thesis) in order to achieve more reliable and comparable results. This method consists of the aspiration of tendon's extremities inside silicon tubes, and their compression in the related anchoring system. This new method has the advantage of reducing stress concentration on tendons over the old method, which consisted of gluing tendon's extremities around spools.

5.3. Future work

In order to advance the results of this study toward clinical treatments, four strategies could be implemented.

5.3.1 Developing tendinopathy using the *ex vivo* model with some modifications

To further validate the efficacy of our suggested combination therapy on tendinopathy, we could develop a tendinopathy model *ex vivo*, with following modifications:

Using SD model *ex vivo*, while changing some parameters such as: species, age, race, or sex of the animal

The results of our study regarding development of moderate tendinopathy, and the effect of applied treatments on moderate tendinopathic tendon, were duplicable for tail tendons of 4-6 months old male Sprague-Dawley rats. Changing the species, tendon, age, race, or sex of the animal might change some of the results, for example the time point of the moderate tendinopathy development or the treatment efficiency. As an example, we observed in preliminary studies that tendons from older rats seem to respond less efficiently to the treatments (data note presented in this thesis). Moreover, it has been suggested that there is a difference between men and women in terms of the risk of

tendinopathy development [32] and also respond to treatments, because alternative mechanisms may be involved[80]. In addition, genetic differences between different races and species might affect their load resistance. For example, HCR rats, which have high endurance running capacity, will probably develop moderate tendinopathy in later time points.

Changing the method of tendinopathy development *ex vivo*

During SD, tendon properties degrade without exposing the tendon to mechanical loading. Therefore, SD models do not represent simultaneous enzymatic and mechanical degradation. For this reason, SD models relate more closely to immobilization scenarios such as casting or rest after surgery rather than overuse injuries. A subject of future studies could be developing overuse (ex: in the bioreactor), or surgically-induced tendinopathy rather than underuse (SD), and testing the combination of PRP and MMPIs on these.

5.3.2 Developing tendinopathy using an *in vivo* model.

Although *ex vivo* methods create well-defined tendinopathy models, *in vivo* models are essential to study the effects of other systems of whole body on tendon pathology and healing and therefore to gain a more complete understanding of the tendinopathy under real conditions. For example, vascularization, which may affect tendon repair, is absent in *ex vivo* models. Therefore, *in vivo* animal studies should be conducted to provide a better understanding of tendinopathy and to test the effect of combination of PRP and MMPIs on different types of injury (SD, overuse, acute).

5.3.3. Developing different stages of tendinopathy *ex vivo* or *in vivo*

Applying our suggested combination therapy on more advanced stages of tendinopathy, *ex vivo* or *in vivo*, could be another subject of future studies. It is possible that the treatment efficiency changes with tendinopathy stages, since the level of damages and cells mechanostat set points also changes.

5.3.4. Modifying amount and/or time point of SL application on *ex vivo* tendinopathy model

In our experiments, exposing damaged tendons to treatments at the same time as applying SL on them, resulted in inferior mechanical results comparing to 10 day non-treated samples.

Applying SL with a delay to other treatments i.e., MMPI and/or PRP, could also be an interesting subject of future studies to improve the treatment strategy for tendinopathy. In this way, MMPI and/or PRP could help develop an ECM which could transfer, and respond to loads more properly, before exposing tendons to SL.

Finally, a more fundamental study to better understand the interaction of cell and ECM would be to applying higher amounts of SL, to reset mechanostat set point of the cells, while allowing some rest periods between loading episodes. According to the literature, this method would cause cell repair response, while minimizing mechanical degradation due to rest periods.

Conclusion (en francais)

Les affections aux tendons sont des problèmes de santé courants. Ils peuvent toucher plusieurs aspects de la vie des gens, des activités quotidiennes aux activités professionnelles. Bien que de nombreuses stratégies aient été suggérées pour traiter les tendinopathies chroniques, aucune n'était complètement satisfaisante. Des modèles animaux de tendinopathies (*in vivo* et *ex vivo*) aiderait non seulement à mieux comprendre la pathologie des lésions tendineuses, mais aussi à trouver un traitement optimal pour cette affection.

Dans cette étude, nous avons décidé de caractériser un modèle *ex vivo*, en tenant compte de notre expertise et de notre équipement en laboratoire. De plus, dans les modèles *ex vivo*, contrairement aux modèles *in vivo*, il est plus facile de développer et de reproduire le modèle de tendinopathie. C'est parce que, dans les modèles *ex vivo*, il n'est pas nécessaire d'interagir avec l'animal de manière récurrente pour le faire s'exercer, par exemple. Par conséquent, ces modèles sont moins chers et prennent moins de temps. Cependant, les modèles *in vivo* pourraient être la prochaine étape après induction de la tendinopathie *ex vivo*, car ils sont plus complets et plus proches du contexte humain.

Parmi toutes les méthodes disponibles pour développer une tendinopathie *ex vivo*, nous avons choisi la privation de chargement mécanique. Bien que les modèles de privation de chargement aient déjà été utilisés pour créer des tendinopathies, il y avait un manque de connaissances concernant le stade modéré des dommages. Le stade modéré de la tendinopathie est crucial, surtout lorsqu'il s'agit d'étudier l'efficacité des traitements. D'une part, le stade précoce de la tendinopathie humaine est souvent asymptomatique[48,65]. En revanche, les tendinopathies avancées peuvent être difficiles à traiter. Par conséquent, une tendinopathie modérée pourrait être un niveau optimal de dommage pour étudier l'efficacité des traitements.

Pour traiter la tendinopathie, une combinaison de PRP et de IMPMs a été proposée dans cette étude. Ces traitements ont été choisies en fonction de leurs résultats prometteurs

dans la littérature. En outre, la modélisation de la réponse mécanobiologique du tendon (RMT),

RMT = Réparation - (dégradation mécanique + dégradation enzymatique)[22] ,

indique que la promotion de la réparation par la PRP et l'inhibition de la dégénération médiée par la métalloprotéase via le IMPM semblent être une approche complémentaire pour retrouver la santé des tendons. Nos résultats indiquent que 4 jours de SD sont nécessaires pour caractériser un modèle modéré de tendinopathie et que la combinaison de IÉ et de PRP est la meilleure par rapport à IÉ, IL, PRP et à la combinaison de IÉ et de PRP.

Appendix

A.1 Matrix metalloproteinases

The enzymes which are capable of breaking down the proteins into their smaller components such as peptides or amino acids are called proteinases. Matrix metalloproteinases (MMPs) are a family of proteinases, responsible for extracellular matrix (ECM) degradation[69]. There are more than 20 MMPs identified which are subdivided on the basis of their substrates into several groups: collagenases, gelatinases, stromelysins, membrane type MMPs (MT-MMPs), and other members that do not fit properly in these groups[99].

The main function of MMPs is to degrade ECM molecules[69]. However ECM degradation could lead to further biological activities which consequently affect non-ECM molecules too[69]. The effect of MMPs on ECM, and non-ECM molecules could be constructive such as: wound healing and bone growth and development, or destructive such as: tumor invasion and arthritis[61].

MMP's activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs). In normal condition, i.e. when MMPs and TIMPs are balanced, MMPs activity results in tissue developing, remodeling and repair. However, when the balance between MMPs and their inhibitors is dysregulated, MMPs activity causes damage in tissue. Therefore, the balance between MMP and TIMP activity is crucial to tissue homeostasis[69].

A.2 Nucleus shape

Figure A.2.1 shows representative of fresh and 10-day SD nucleus stained with DAPI (DAPI, Sigma-Aldrich, 62248, 1 µg/ml).

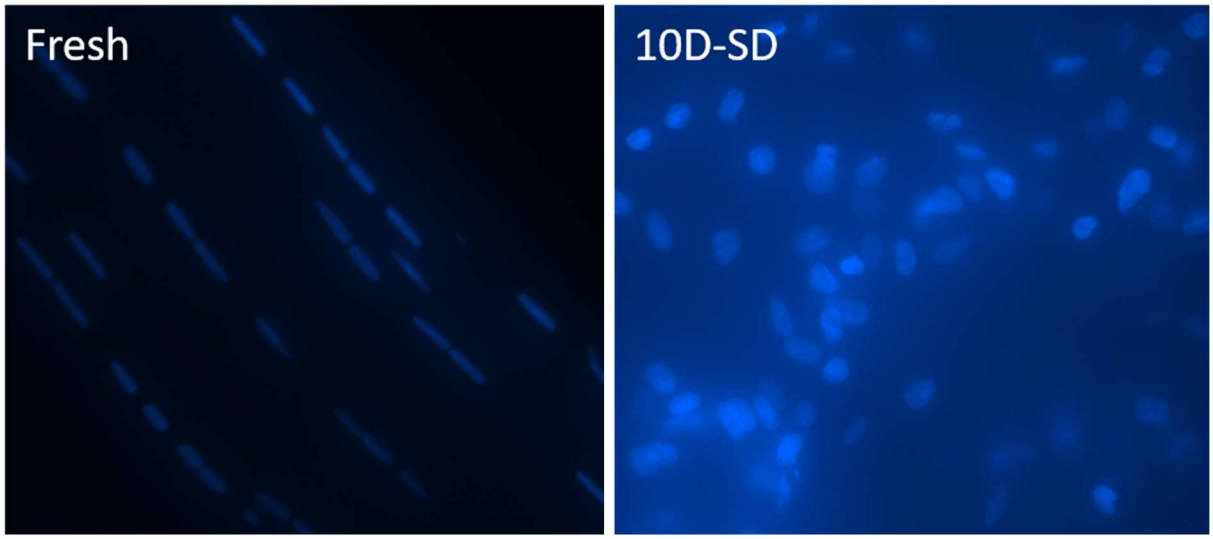

Figure A.2.1 Typical micrographs of longitudinal sections of fresh and 10 day SD tendons stained with DAPI. Bar=100 μm fresh and 10-day SD tendons of DAPI stained nucleus.

Using Image-Pro Plus software, the nucleus shape was traced automatically, based on the contrast. The roundness of nucleus was measured using "roundness" tool with the following formula:

$$Nucleus\ roundness\ = \frac{Perimeter\ of\ nucleus^2}{4*\pi*Area\ of\ nucleus} \quad \text{(A.1)}$$

A value of 1 indicates a perfect circle, whereas higher values indicate more elongated shapes.

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References

1. Abreu, E. L., D. Leigh, and K. A. Derwin. Effect of altered mechanical load conditions on the structure and function of cultured tendon fascicles. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 26:364–373, 2008.

2. Andres, B. M., and G. A. C. Murrell. Treatment of tendinopathy: what works, what does not, and what is on the horizon. *Clin. Orthop.* 466:1539–1554, 2008.

3. Anitua, E., I. Andía, M. Sanchez, J. Azofra, M. del Mar Zalduendo, M. de la Fuente, P. Nurden, and A. T. Nurden. Autologous preparations rich in growth factors promote proliferation and induce VEGF and HGF production by human tendon cells in culture. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 23:281–286, 2005.

4. Arnoczky, S. P., M. Lavagnino, and M. Egerbacher. The mechanobiological aetiopathogenesis of tendinopathy: is it the over-stimulation or the under-stimulation of tendon cells? *Int. J. Exp. Pathol.* 88:217–226, 2007.

5. Arnoczky, S. P., M. Lavagnino, M. Egerbacher, O. Caballero, and K. Gardner. Matrix metalloproteinase inhibitors prevent a decrease in the mechanical properties of stress-deprived tendons: an in vitro experimental study. *Am. J. Sports Med.* 35:763–769, 2007.

6. Arnoczky, S. P., M. Lavagnino, M. Egerbacher, O. Caballero, K. Gardner, and M. A. Shender. Loss of homeostatic strain alters mechanostat “set point” of tendon cells in vitro. *Clin. Orthop.* 466:1583–1591, 2008.

7. Arnoczky, S. P., M. Lavagnino, J. H. Whallon, and A. Hoonjan. In situ cell nucleus deformation in tendons under tensile load; a morphological analysis using confocal laser microscopy. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 20:29–35, 2002.

8. Arnoczky, S. P., T. Tian, M. Lavagnino, and K. Gardner. Ex vivo static tensile loading inhibits MMP-1 expression in rat tail tendon cells through a cytoskeletally based mechanotransduction mechanism. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 22:328–333, 2004.

9. Aubin, F., L. Javaudin, and P. Rochcongar. Case report of aprotinin in Achilles tendinopathies with athletes. *J. Pharm. Clin.* 16:270–273, 1997.

10. Baker, A. H., D. R. Edwards, and G. Murphy. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J. Cell Sci.* 115:3719–3727, 2002.

11. Baksh, N., C. P. Hannon, C. D. Murawski, N. A. Smyth, and J. G. Kennedy. Platelet-rich plasma in tendon models: a systematic review of basic science literature. *Arthrosc. J. Arthrosc. Relat. Surg. Off. Publ. Arthrosc. Assoc. N. Am. Int. Arthrosc. Assoc.* 29:596–607, 2013.

12. Baragi, V. M. *et al.* A new class of potent matrix metalloproteinase 13 inhibitors for potential treatment of osteoarthritis: Evidence of histologic and clinical efficacy without musculoskeletal toxicity in rat models. *Arthritis Rheum.* 60:2008–2018, 2009.

13. Bedi, A., A. J. S. Fox, D. Kovacevic, X.-H. Deng, R. F. Warren, and S. A. Rodeo. Doxycycline-mediated inhibition of matrix metalloproteinases improves healing after rotator cuff repair. *Am. J. Sports Med.* 38:308–317, 2010.

14. Bedi, A., D. Kovacevic, C. Hettrich, L. V. Gulotta, J. R. Ehteshami, R. F. Warren, and S. A. Rodeo. The effect of matrix metalloproteinase inhibition on tendon-to-bone healing in a rotator cuff repair model. *J. Shoulder Elbow Surg.* 19:384–391, 2010.

15. Bisciotti, G. N., and P. Volpi. Tendonitis, Tendinosis, or Tendinopathy? In: The Lower Limb Tendinopathies. Springer, Cham, 2016, pp. 1–19.doi:10.1007/978-3-319-33234-5_1

16. Bosch, G., H. T. M. van Schie, M. W. de Groot, J. A. Cadby, C. H. A. van de Lest, A. Barneveld, and P. R. van Weeren. Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor tendons: A placebo-controlled experimental study. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 28:211–217, 2010.

17. Brown, R., J. Orchard, M. Kinchington, A. Hooper, and G. Nalder. Aprotinin in the management of Achilles tendinopathy: a randomised controlled trial. *Br. J. Sports Med.* 40:275–279, 2006.

18. Bruneau, A., N. Champagne, P. Cousineau-Pelletier, G. Parent, and E. Langelier. Preparation of Rat Tail Tendons for Biomechanical and Mechanobiological Studies. *J. Vis. Exp. JoVE* , 2010.doi:10.3791/2176

19. Capasso, G., N. Maffulli, V. Testa, and A. Sgambato. Preliminary results with peritendinous protease inhibitor injections in the management of Achilles tendinitis. *J. Sports Traumatol. Relat. Res.* 15:37–42, 1993.

20. Chen, L., J.-P. Liu, K.-L. Tang, Q. Wang, G.-D. Wang, X.-H. Cai, and X.-M. Liu. Tendon derived stem cells promote platelet-rich plasma healing in collagenase-induced rat achilles tendinopathy. *Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol.* 34:2153–2168, 2014.

21. Chimenti, R. L., C. C. Cychosz, M. M. Hall, and P. Phisitkul. Current Concepts Review Update: Insertional Achilles Tendinopathy. *Foot Ankle Int.* 38:1160–1169, 2017.

22. Cousineau-Pelletier, P., and E. Langelier. Relative contributions of mechanical degradation, enzymatic degradation, and repair of the extracellular matrix on the response of tendons when subjected to under- and over- mechanical stimulations in vitro. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 28:204–210, 2010.

23. Davis, M. E., J. P. Gumucio, K. B. Sugg, A. Bedi, and C. L. Mendias. MMP inhibition as a potential method to augment the healing of skeletal muscle and tendon extracellular matrix. *J. Appl. Physiol.* 115:884–891, 2013.

24. Del Buono, A., F. Oliva, L. Osti, and N. Maffulli. Metalloproteases and tendinopathy. *Muscles Ligaments Tendons J.* 3:51–57, 2013.

25. Demirag, B., B. Sarisozen, O. Ozer, T. Kaplan, and C. Ozturk. Enhancement of tendon-bone healing of anterior cruciate ligament grafts by blockage of matrix metalloproteinases. *J. Bone Joint Surg. Am.* 87:2401–2410, 2005.

26. Devkota, A. C., M. Tsuzaki, L. C. Almekinders, A. J. Banes, and P. S. Weinhold. Distributing a fixed amount of cyclic loading to tendon explants over longer periods induces greater cellular and mechanical responses. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 25:1078–1086, 2007.

27. Dirks, R. C., and S. J. Warden. Models for the study of tendinopathy. *J. Musculoskelet. Neuronal Interact.* 11:141–149, 2011.

28. Docking, S., T. Samiric, E. Scase, C. Purdam, and J. Cook. Relationship between compressive loading and ECM changes in tendons. *Muscles Ligaments Tendons J.* 3:7–11, 2013.

29. Egerbacher, M., S. P. Arnoczky, O. Caballero, M. Lavagnino, and K. L. Gardner. Loss of homeostatic tension induces apoptosis in tendon cells: an in vitro study. *Clin. Orthop.* 466:1562–1568, 2008.

30. Egerbacher, M Arnoczky, SP; Gardner, K; Caballero, O; Gartner, J. STRESS-DEPRIVATION OF TENDONS RESULTS IN ALTERATIONS IN THE INTEGRIN PROFILE AND PERICELLULAR MATRIX OF TENDON CELLS. , 2006.

31. Federer, A. E., J. R. Steele, T. J. Dekker, J. L. Liles, and S. B. Adams. Tendonitis and Tendinopathy: What Are They and How Do They Evolve? *Foot Ankle Clin.* 22:665–676, 2017.

32. Frizziero, A., F. Vittadini, G. Gasparre, and S. Masiero. Impact of oestrogen deficiency and aging on tendon: concise review. *Muscles Ligaments Tendons J.* 4:324–328, 2014.

33. Galloway, M. T., A. L. Lalley, and J. T. Shearn. The role of mechanical loading in tendon development, maintenance, injury, and repair. *J. Bone Joint Surg. Am.* 95:1620–1628, 2013.

34. Gardner, K., S. P. Arnoczky, O. Caballero, and M. Lavagnino. The effect of stress-deprivation and cyclic loading on the TIMP/MMP ratio in tendon cells: an in vitro experimental study. *Disabil. Rehabil.* 30:1523–1529, 2008.

35. Gautam, V., S. Verma, S. Batra, N. Bhatnagar, and S. Arora. Platelet-Rich Plasma versus Corticosteroid Injection for Recalcitrant Lateral Epicondylitis: Clinical and Ultrasonographic Evaluation. *J. Orthop. Surg.* 23:1–5, 2015.

36. Giusti, I., S. D'Ascenzo, A. Mancò, G. Di Stefano, M. Di Francesco, A. Rughetti, A. Dal Mas, G. Properzi, V. Calvisi, and V. Dolo. Platelet Concentration in Platelet-Rich Plasma Affects Tenocyte Behavior In Vitro. , 2014.doi:10.1155/2014/630870

37. Hannafin, J. A., S. P. Arnoczky, A. Hoonjan, and P. A. Torzilli. Effect of stress deprivation and cyclic tensile loading on the material and morphologic properties of canine flexor digitorum profundus tendon: an in vitro study. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 13:907–914, 1995.

38. Jafari, L., Y. Lemieux-Laneuville, D. Gagnon, and E. Langelier. Low amplitude characterization tests conducted at regular intervals can affect tendon mechanobiological response. *Ann. Biomed. Eng.* 42:589–599, 2014.

39. Jafari L, Savard M, Gobeil F, Langelier E. Characterization of moderate tendinopathy in ex vivo stress-deprived rat tail tendons. *J. BMC (Conditionally acceptable)* , 2018.

40. James, R., G. Kesturu, G. Balian, and A. B. Chhabra. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *J. Hand Surg.* 33:102–112, 2008.

41. de Jonge, S., R. J. de Vos, A. Weir, H. T. M. van Schie, S. M. A. Bierma-Zeinstra, J. A. N. Verhaar, H. Weinans, and J. L. Tol. One-year follow-up of platelet-rich plasma treatment in chronic Achilles tendinopathy: a double-blind randomized placebo-controlled trial. *Am. J. Sports Med.* 39:1623–1629, 2011.

42. Kalson, N. S., D. F. Holmes, Z. Kapacee, I. Otermin, Y. Lu, R. A. Ennos, E. G. Canty-Laird, and K. E. Kadler. An experimental model for studying the biomechanics of embryonic tendon: Evidence that the development of mechanical properties depends on the actinomyosin machinery. *Matrix Biol. J. Int. Soc. Matrix Biol.* 29:678–689, 2010.

43. Kaux, J.-F., B. Forthomme, C. L. Goff, J.-M. Crielaard, and J.-L. Croisier. Current opinions on tendinopathy. *J. Sports Sci. Med.* 10:238–253, 2011.

44. Kessler, M. W., J. Barr, R. Greenwald, L. B. Lane, J. S. Dines, D. M. Dines, M. C. Drakos, D. A. Grande, and N. O. Chahine. Enhancement of Achilles tendon repair mediated by matrix metalloproteinase inhibition via systemic administration of doxycycline. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 32:500–506, 2014.

45. Killian, M. L., L. Cavinatto, L. M. Galatz, and S. Thomopoulos. THE ROLE OF MECHANOBIOLOGY IN TENDON HEALING. *J. Shoulder Elbow Surg.* 21:228–237, 2012.

46. Kjaer, M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol. Rev.* 84:649–698, 2004.

47. Kon, E., G. Filardo, M. Delcogliano, M. L. Presti, A. Russo, A. Bondi, A. Di Martino, A. Cenacchi, P. M. Fornasari, and M. Marcacci. Platelet-rich plasma: new clinical application: a pilot study for treatment of jumper's knee. *Injury* 40:598–603, 2009.

48. Lake, S. P., H. L. Ansorge, and L. J. Soslowsky. Animal models of tendinopathy. *Disabil. Rehabil.* 30:1530–1541, 2008.

49. Lavagnino, M., S. P. S. P. Arnoczky, K. Frank, and T. Tian. Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons. *J. Biomech.* 38:69–75, 2005.

50. Lavagnino, M., S. P. Arnoczky, T. Tian, and Z. Vaupel. Effect of amplitude and frequency of cyclic tensile strain on the inhibition of MMP-1 mRNA expression in tendon cells: an in vitro study. *Connect. Tissue Res.* 44:181–187, 2003.

51. Lee, J.-Y., K. Yoon, Y. Yi, C.-H. Park, J.-S. Lee, K.-H. Seo, Y. S. Park, and Y.-T. Lee. Long-Term Outcome and Factors Affecting Prognosis of Extracorporeal Shockwave Therapy for Chronic Refractory Achilles Tendinopathy. *Ann. Rehabil. Med.* 41:42–50, 2017.

52. Li, H., F. Lindenmeyer, C. Grenet, P. Opolon, S. Menashi, C. Soria, P. Yeh, M. Perricaudet, and H. Lu. AdTIMP-2 inhibits tumor growth, angiogenesis, and metastasis, and prolongs survival in mice. *Hum. Gene Ther.* 12:515–526, 2001.

53. Liu, Y., H. S. Ramanath, and D.-A. Wang. Tendon tissue engineering using scaffold enhancing strategies. *Trends Biotechnol.* 26:201–209, 2008.

54. Loiselle, A. E., G. A. Bragdon, J. A. Jacobson, S. Hasslund, Z. E. Cortes, E. M. Schwarz, D. J. Mitten, H. A. Awad, and R. J. O'Keefe. Remodeling of murine intrasynovial tendon adhesions following injury: MMP and neotendon gene expression. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 27:833–840, 2009.

55. Lui, P. P. Y., N. Maffulli, C. Rolf, and R. K. W. Smith. What are the validated animal models for tendinopathy? *Scand. J. Med. Sci. Sports* 21:3–17, 2011.

56. Lyras, D., K. Kazakos, D. Verettas, A. Polychronidis, C. Simopoulos, S. Botaitis, G. Agrogiannis, A. Kokka, and E. Patsouris. Immunohistochemical study of angiogenesis after local administration of platelet-rich plasma in a patellar tendon defect. *Int. Orthop.* 34:143–148, 2010.

57. Lyras, D. N., K. Kazakos, G. Georgiadis, G. Mazis, R. Middleton, S. Richards, D. O'Connor, and G. Agrogiannis. Does a single application of PRP alter the expression of IGF-I in the early phase of tendon healing? *J. Foot Ankle Surg. Off. Publ. Am. Coll. Foot Ankle Surg.* 50:276–282, 2011.

58. Lyras, D. N., K. Kazakos, D. Verettas, S. Botaitis, G. Agrogiannis, A. Kokka, M. Pitiakoudis, and A. Kotzakaris. The effect of platelet-rich plasma gel in the early phase of patellar tendon healing. *Arch. Orthop. Trauma Surg.* 129:1577–1582, 2009.

59. Lyras, D. N., K. Kazakos, D. Verettas, A. Polychronidis, M. Tryfonidis, S. Botaitis, G. Agrogiannis, C. Simopoulos, A. Kokka, and E. Patsouris. The influence of platelet-rich plasma on angiogenesis during the early phase of tendon healing. *Foot Ankle Int.* 30:1101–1106, 2009.

60. Maffulli, N., U. G. Longo, M. Loppini, F. Spiezia, and V. Denaro. New options in the management of tendinopathy. *Open Access J. Sports Med.* 1:29–37, 2010.

61. Magra, M., and N. Maffulli. Molecular events in tendinopathy: a role for metalloproteases. *Foot Ankle Clin.* 10:267–277, 2005.

62. Maquoi, E., N. E. Sounni, L. Devy, F. Olivier, F. Frankenne, H.-W. Krell, F. Grams, J.-M. Foidart, and A. Noël. Anti-Invasive, Antitumoral, and Antiangiogenic Efficacy of a Pyrimidine-2,4,6-trione Derivative, an Orally Active and Selective Matrix Metalloproteinases Inhibitor. *Clin. Cancer Res.* 10:4038–4047, 2004.

63. Mautner, K., R. E. Colberg, G. Malanga, J. P. Borg-Stein, K. G. Harmon, A. S. Dharamsi, S. Chu, and P. Homer. Outcomes after ultrasound-guided platelet-rich plasma injections for chronic tendinopathy: a multicenter, retrospective review. *PM R* 5:169–175, 2013.

64. McNeilly, C. M., A. J. Banes, M. Benjamin, and J. R. Ralphs. Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions. *J. Anat.* 189 (Pt 3):593–600, 1996.

65. Millar, N. L., G. A. C. Murrell, and I. B. McInnes. Inflammatory mechanisms in tendinopathy - towards translation. *Nat. Rev. Rheumatol.* 13:110–122, 2017.

66. Mishra, A., and T. Pavelko. Treatment of chronic elbow tendinosis with buffered platelet-rich plasma. *Am. J. Sports Med.* 34:1774–1778, 2006.

67. Morizaki, Y., C. Zhao, K.-N. An, and P. C. Amadio. The Effects of Platelet-Rich Plasma on Bone Marrow Stromal Cell Transplants for Tendon Healing In Vitro. *J. Hand Surg.* 35:1833–1841, 2010.

68. de Mos, M., A. E. van der Windt, H. Jahr, H. T. M. van Schie, H. Weinans, J. A. N. Verhaar, and G. J. V. M. van Osch. Can platelet-rich plasma enhance tendon repair? A cell culture study. *Am. J. Sports Med.* 36:1171–1178, 2008.

69. Nagase, H., R. Visse, and G. Murphy. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* 69:562–573, 2006.

70. Nguyen, Q. T., J. B. Norelli, A. Graver, C. Ekstein, J. Schwartz, F. Chowdhury, M. C. Drakos, D. A. Grande, and N. O. Chahine. Therapeutic Effects of Doxycycline on the Quality of Repaired and Unrepaired Achilles Tendons. *Am. J. Sports Med.* 45:2872–2881, 2017.

71. Nordin, M., and V. H. Frankel. Basic Biomechanics of the Musculoskeletal System. Lippincott Williams & Wilkins, 2001, 498 pp.

72. Nourissat, G., F. Berenbaum, and D. Duprez. Tendon injury: from biology to tendon repair. *Nat. Rev. Rheumatol.* 11:223–233, 2015.

73. Orchard, J., A. Massey, R. Brown, A. Cardon-Dunbar, and J. Hofmann. Successful management of tendinopathy with injections of the MMP-inhibitor aprotinin. *Clin. Orthop.* 466:1625–1632, 2008.

74. Orner, C. A., M. B. Geary, W. C. Hammert, R. J. O'Keefe, and A. E. Loiselle. Low-Dose and Short-Duration Matrix Metalloproteinase 9 Inhibition Does Not Affect Adhesion Formation during Murine Flexor Tendon Healing. *Plast. Reconstr. Surg.* 137:545e–553e, 2016.

75. Oshiro, W., J. Lou, X. Xing, Y. Tu, and P. R. Manske. Flexor tendon healing in the rat: a histologic and gene expression study. *J. Hand Surg.* 28:814–823, 2003.

76. Parent, G., M. Cyr, F. Desbiens-Blais, and È. Langelier. Bias and precision of algorithms in estimating the cross-sectional area of rat tail tendons. *Meas. Sci. Technol.* 21:125802, 2010.

77. Parent, G., N. Huppé, and E. Langelier. Low stress tendon fatigue is a relatively rapid process in the context of overuse injuries. *Ann. Biomed. Eng.* 39:1535–1545, 2011.

78. Pasternak, B., and P. Aspenberg. Metalloproteinases and their inhibitors-diagnostic and therapeutic opportunities in orthopedics. *Acta Orthop.* 80:693–703, 2009.

79. Pasternak, B., M. Fellenius, and P. Aspenberg. Doxycycline impairs tendon repair in rats. *Acta Orthop. Belg.* 72:756–760, 2006.

80. Pease, L. I., P. D. Clegg, C. J. Proctor, D. J. Shanley, S. J. Cockell, and M. J. Peffers. Cross platform analysis of transcriptomic data identifies ageing has distinct and opposite effects on tendon in males and females. *Sci. Rep.* 7:14443, 2017.

81. Peerbooms, J. C., J. Sluimer, D. J. Bruijn, and T. Gosens. Positive effect of an autologous platelet concentrate in lateral epicondylitis in a double-blind randomized controlled trial: platelet-rich plasma versus corticosteroid injection with a 1-year follow-up. *Am. J. Sports Med.* 38:255–262, 2010.

82. Pifer, M. A., T. Maerz, K. C. Baker, and K. Anderson. Matrix Metalloproteinase Content and Activity in Low-Platelet, Low-Leukocyte and High-Platelet, High-Leukocyte

Platelet Rich Plasma (PRP) and the Biologic Response to PRP by Human Ligament Fibroblasts. *Am. J. Sports Med.* 42:1211–1218, 2014.

83. Rees, J. D., M. Stride, and A. Scott. Tendons--time to revisit inflammation. *Br. J. Sports Med.* 48:1553–1557, 2014.

84. Rees, J. D., A. M. Wilson, and R. L. Wolman. Current concepts in the management of tendon disorders. *Rheumatol. Oxf. Engl.* 45:508–521, 2006.

85. Reno et al. Rapid Isolation of Total RNA from Small Samples of Hypocellular, Dense Connective Tissues. *BioTechniques* 22:1082–1086, 1997.

86. Riley, G. Tendinopathy--from basic science to treatment. *Nat. Clin. Pract. Rheumatol.* 4:82–89, 2008.

87. Rodeo, S. A., D. Delos, R. J. Williams, R. S. Adler, A. Pearle, and R. F. Warren. The Effect of Platelet-Rich Fibrin Matrix on Rotator Cuff Tendon Healing: A Prospective, Randomized Clinical Study. *Am. J. Sports Med.* 40:1234–1241, 2012.

88. Savard Martin, Barbaz David, Bélanger Simon, Müller-Esterl Werner, Bkaily Ghassan, D'orléans-Juste Pedro, Coté Jérôme, Bovenzi Veronica, and Gobeil Fernand. Expression of endogenous nuclear bradykinin B2 receptors mediating signaling in immediate early gene activation. *J. Cell. Physiol.* 216:234–244, 2008.

89. Schnabel, L. V., H. O. Mohammed, B. J. Miller, W. G. McDermott, M. S. Jacobson, K. S. Santangelo, and L. A. Fortier. Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 25:230–240, 2007.

90. Sharma, P., and N. Maffulli. Tendon injury and tendinopathy: healing and repair. *J. Bone Joint Surg. Am.* 87:187–202, 2005.

91. Sharma, P., and N. Maffulli. Biology of tendon injury: healing, modeling and remodeling. *J. Musculoskelet. Neuronal Interact.* 6:181–190, 2006.

92. Silva, A., and R. Sampaio. Anatomic ACL reconstruction: does the platelet-rich plasma accelerate tendon healing? *Knee Surg. Sports Traumatol. Arthrosc.* 17:676–682, 2009.

93. Spang, J. T., T. Tischer, G. M. Salzmann, T. Winkler, R. Burgkart, G. Wexel, and A. B. Imhoff. Platelet concentrate vs. saline in a rat patellar tendon healing model. *Knee Surg. Sports Traumatol. Arthrosc.* 19:495–502, 2011.

94. Taylor, D. W., M. Petrera, M. Hendry, and J. S. Theodoropoulos. A systematic review of the use of platelet-rich plasma in sports medicine as a new treatment for tendon and ligament injuries. *Clin. J. Sport Med. Off. J. Can. Acad. Sport Med.* 21:344–352, 2011.

95. Tohidnezhad, M., D. Varoga, C. J. Wruck, L. O. Brandenburg, A. Seekamp, M. Shakibaei, T. T. Sönmez, T. Pufe, and S. Lippross. Platelet-released growth factors can accelerate tenocyte proliferation and activate the anti-oxidant response element. *Histochem. Cell Biol.* 135:453–460, 2011.

96. Uchida, H., H. Tohyama, K. Nagashima, Y. Ohba, H. Matsumoto, Y. Toyama, and K. Yasuda. Stress deprivation simultaneously induces over-expression of interleukin-1beta, tumor necrosis factor-alpha, and transforming growth factor-beta in fibroblasts and mechanical deterioration of the tissue in the patellar tendon. *J. Biomech.* 38:791–798, 2005.

97. Virchenko, O., and P. Aspenberg. How can one platelet injection after tendon injury lead to a stronger tendon after 4 weeks?: Interplay between early regeneration and mechanical stimulation. *Acta Orthop.* 77:806–812, 2006.

98. Voleti, P. B., M. R. Buckley, and L. J. Soslowsky. Tendon healing: repair and regeneration. *Annu. Rev. Biomed. Eng.* 14:47–71, 2012.

99. Vu, T. H., and Z. Werb. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* 14:2123–2133, 2000.

100. Wang, J. H.-C. Mechanobiology of tendon. *J. Biomech.* 39:1563–1582, 2006.

101. Wang, J. H.-C., M. I. Iosifidis, and F. H. Fu. Biomechanical basis for tendinopathy. *Clin. Orthop.* 443:320–332, 2006.

102. Wang, T., Z. Lin, M. Ni, C. Thien, R. E. Day, B. Gardiner, J. Rubenson, T. B. Kirk, D. W. Smith, A. Wang, D. G. Lloyd, Y. Wang, Q. Zheng, and M. H. Zheng. Cyclic mechanical stimulation rescues achilles tendon from degeneration in a bioreactor system. *J. Orthop. Res. Off. Publ. Orthop. Res. Soc.* 33:1888–1896, 2015.

103. Wesner, M., T. Defreitas, H. Bredy, L. Pothier, Z. Qin, A. B. McKillop, and D. P. Gross. A Pilot Study Evaluating the Effectiveness of Platelet-Rich Plasma Therapy for Treating Degenerative Tendinopathies: A Randomized Control Trial with Synchronous Observational Cohort. *PLOS ONE* 11:e0147842, 2016.

104. Wnuk, T., J. Blacha, T. Mazurkiewicz, G. Olchowik, and M. Chyżyńska. The mechanical and histological estimation of calcaneal tendon callus in rats after PRP injection. *Pol. Orthop. Traumatol.* 77:5–9, 2012.

105. Wroblewski, A. P., H. A. Mejia, and V. J. Wright. Application of Platelet-Rich Plasma to Enhance Tissue Repair. *Oper. Tech. Orthop.* 20:98–105, 2010.

106. Xu, Y., and G. A. C. Murrell. The basic science of tendinopathy. *Clin. Orthop.* 466:1528–1538, 2008.

107. Yamamoto, E., W. Iwanaga, H. Miyazaki, and K. Hayashi. Effects of static stress on the mechanical properties of cultured collagen fascicles from the rabbit patellar tendon. *J. Biomech. Eng.* 124:85–93, 2002.

108. Yamamoto, N., K. Ohno, K. Hayashi, H. Kuriyama, K. Yasuda, and K. Kaneda. Effects of stress shielding on the mechanical properties of rabbit patellar tendon. *J. Biomech. Eng.* 115:23–28, 1993.

109. Ye, S. Putative targeting of matrix metalloproteinase-8 in atherosclerosis. *Pharmacol. Ther.* 147:111–122, 2015.

110. Yuan, J., M.-X. Wang, and G. A. C. Murrell. Cell death and tendinopathy. *Clin. Sports Med.* 22:693–701, 2003.

111. Zabrzyński, J., Ł. Łapaj, Ł. Paczesny, A. Zabrzyńska, and D. Grzanka. Tendon - function related structure, simple healing process and mysterious ageing. *Folia Morphol.* , 2018.doi:10.5603/FM.a2018.0006

112. Zhang, J., and J. H.-C. Wang. PRP treatment effects on degenerative tendinopathy - an in vitro model study. *Muscles Ligaments Tendons J.* 4:10–17, 2014.

113. Zhou, Y., J. Zhang, H. Wu, M. V. Hogan, and J. H.-C. Wang. The differential effects of leukocyte-containing and pure platelet-rich plasma (PRP) on tendon stem/progenitor cells - implications of PRP application for the clinical treatment of tendon injuries. *Stem Cell Res. Ther.* 6:173, 2015.

114. Tendinopathies of the wrist and hand - ScienceDirectat <https://www.sciencedirect.com/science/article/pii/B9780702035289000261>